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Determination of free and encapsulated oligonucleotides in liposome formulated drug product

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Abstract

Liposomes have been recognized as new delivery vehicles for peptide and oligonucleotide drugs, offering effective drug protection and influencing drug distribution from the circulation to tissues. To ensure consistent formulation behavior and drug distribution, the amounts of free and encapsulated active pharmaceutical ingredient in the liposome formulation must be determined. A simple and reliable method has been developed for the determination of free and encapsulated oligonucleotide drugs in liposomes. Capillary electrophoresis in entangled polyacrylamide solution was optimized for the analysis. Liposome samples were treated to release encapsulated oligonucleotide so that total concentration could be determined, and untreated liposomes were applied directly to capillary column to determine the fraction of free oligonucleotides. Recoveries are 96–105% and relative standard deviations are generally 2–3%. Multiple liposome types were analyzed with satisfactory results.

Keywords: Determination; Capillary electrophoresis; Oligonucleotide drug; Liposome formulation

1. Introduction

Antisense oligonucleotide drugs are being developed as therapies for human diseases ranging from viral infection to inflammatory disease, to cancer [1]. The specific binding of oligonucleotides to their complementary RNA targets by Watson–Crick hybridization can inhibit RNA splicing [2] and mRNA translation [3], and permits degradation of the bound mRNA by endogenous RNase H [4]. Phosphorothioate oligonucleotides are first-generation antisense drugs that are structural analogs of natural DNA modified to enhance nuclease resistance while maintaining RNase H substrate activity and hybridization to the target mRNA [5]. The last 3 years have seen great progress in the development of multiple therapeutic antisense oligonucleotide delivery systems for the intravenous, topical, inhalation, and oral routes, including commercialization of the first antisense drug product. Among these, intravenous formulations affording long circulation times and concomitantly increased drug distribution to tissues are of interest, including liposome-based systems.

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Liposomes are well-recognized intravenous drug delivery vehicles as demonstrated by several drug product regulatory approvals [6–9]. The most useful liposomal drug delivery systems are fully biodegradable, highly biocompatible and easy to modify. Functionally, liposomes can: (1) protect drug remaining with the vesicles; (2) increase drug uptake by tissues into which liposomes distribute; (3) widen dose intervals by increasing drug circulation times; and (4) facilitate drug uptake by cells [6,8]. Typical liposome formulation parameters include lipid composition, vesicle size, lipid membrane fluidity, surface charge, cholesterol content and steric stabilization. The wide range of variable parameters makes liposomes readily modifiable to improve their properties for specific tissue delivery while minimizing drug systemic toxicity. Other factors contributing to the increasing success of liposome-based drug delivery are availability of low cost purity lipids, efficient drug entrapment methods, methods for large-scale manufacture, acceptable toxicities, and growing clinical experience.

Liposome formluation has two basic requirements: (1) minimal batch to batch variability; (2) acceptable stability without significant aggregation or loss of entrapped drug. Hence, analytical methods are needed to determine free and encapsulated drug levels in liposomal formulations.

Few methods have been developed for the analysis of liposomally formulated oligonucleotides. Confocal microscopy was used to assess uptake of liposome encapsulated, fluorescently labeled oligonucleotides [10]. Light and electron microscopies were employed to determine the cellular uptake and intracellular distribution of encapsulated oligonucleotides [8]. Reverse transcriptase assays and the polymerase chain reaction (PCR) were applied to assess antiviral activity of both free and encapsulated oligonucleotides [9]. To the authors' knowledge, however, no method has been developed for the determination of oligonucleotides in liposome formulation. Development of a reliable method to meet this need is reported.

Capillary electrophoresis in entangled polyacrylamide solution was used for the analysis. The liposome sample was processed by solvent extraction or detergent disruption to release encapsulated oligonucleotide, followed by analysis by capillary electrophoresis to determine total oligonucleotide present. Intact liposomes were injected directly into the capillary to selectively determine free oligonucleotides. Due to the zero net charge of the encapsulated solution and the large size of the liposome particle, only external oligonucleotide migrates within the column. For capillary electrophoresis, an internal standard was added to liposome samples for improved quantification. Encapsulated oligonucleotide concentration is calculated base on results of the total and free oligonucleotide determinations. The method is simple and reliable for the determination of free and encapsulated oligonucleotide and has satisfactory accuracy and precision.

2. Material and methods

2.1. Instruments

A Beckman P/ACE 5000 instrument (Fullerton, CA) was used for capillary electrophoresis. Beckman eCAP™ ssDNA R-100 Gel and a corresponding Beckman eCAP™ DNA capillary column were used for analysis of liposome samples. The entangled polyacrylamide solution was filled into the column under high pressure and the column was equilibrated once with electric field before sample analysis. After ~ 15 analysis runs, new entangled polymer solution was filled into the column for the replacement. Approximately ten refill cycles can be made for each capillary column.

2.2. Determination of total oligonucleotide by capillary electrophoresis

2.2.1. Extraction of total oligonucleotides from liposomes

A 500 μl liposome solution sample was transferred into a microcentrifuge tube and 500 μl of extractant (phenol:chloroform:isoamyl alcohol in 25:24:1 (v/v)) was added. The two phases were

mixed by vortex for 1 min and the microcentrifuge tube was centrifuged at 13 000 rpm for 5 min. A 400 μl of the aqueous solution (upper layer) was transferred into another microcentrifuge tube containing 400 μl of the extractant and the mixing and centrifugation processes were repeated. Finally, 250 μl of the aqueous solution was transported into another microcentrifuge tube for analysis. The solution was first diluted with purified water to about 1 mg ml^{-1} of the oligonucleotide concentration. Then, 10 μl of the diluted solution and 50 μl of the internal standard solution, T27 (50 μg ml^{-1}), was added and diluted to 1 ml in a volumetric flask with purified water.

2.2.2. Disruption of the liposome structure by detergent

For the non-extraction procedure, the liposome sample was first diluted to about 1 mg ml^{-1} oligonucleotide concentration. A total of 10 μl of the diluted solution and 50 μl of the internal standard solution, T27 (50 μg ml^{-1}), were added and diluted to 1 ml in a volumetric flask with 3% Triton X-100, which acts to destroy liposome structure and release the oligonucleotides.

2.3. Capillary electrophoresis

Total oligonucleotide concentration was determined by capillary electrophoresis of the sample solution (prepared above) against the reference standard. Standard solution was prepared by adding 10 μl of the 1 mg ml^{-1} of secondary formulated reference standard and 50 μl of the internal standard into 1 mL volumetric flask and diluted to 1 ml with either purified water for extracted samples or 3% Triton X-100 for detergent treated samples.

For the determination of free oligonucleotide in the liposome solution, 1 ml of the liposome solution and 50 μl of the internal standard, T27, were combined and mixed. Capillary electrophoresis was performed on sample solutions against a reference standard prepared by mixing 1 ml of the 10 μg ml^{-1} secondary formulated standard with 50 μl of the internal standard solution, T27.

3. Results and discussion

Encapsulation efficiency for oligonucleotides in liposomes is a very important formulation parameter. After liposomes are made, free (unencapsulated) oligonucleotides are removed by dialysis. A small portion of free oligonucleotide remains, however. Stability of liposome solutions is likewise an issue: encapsulated oligonucleotides should remain inside the vesicle for a relatively long time without leakage. A reliable and accurate method for determination of free and encapsulated oligonucleotides in liposome formulated drug products is therefore needed for in-process control, product release assays, and stability testing. Fig. 1 illustrated the analysis process. As negatively charged oligonucleotides are entrapped in liposomes along with positively charged counterions such as sodium and potassium, net charge inside the liposome is zero. For conventional liposomes made of neutral molecules or for cationic liposomes the net surface charges are zero or positive. Liposomes will either not move at all in an electric field or will migrate in the opposite direction from negatively charged oligonucleotides. Therefore, direct capillary electrophoresis can be used to determine free oligonucleotides migrating to the anode. An alternative method to determine unencapsulated oligonucleotides is filtration on Centricon-100 (100 000 MW) filters. Liposomes are retained while free oligonucleotide pass through the filter and are collected for determination by capillary electrophoresis. For determination of total oligonucleotides in liposome solutions, vesicles are first disrupted in phenol–chloroform or with a detergent such as Triton X-100 to release internal oligonucleotides.

3.1. Capillary electrophoresis

Capillary electrophoresis is an excellent separation method for oligonucleotides [11–19]. The method can readily resolve oligonucleotides of the same sequence with single base differences in length. Analysis times are the same or shorter than those for HPLC. Quantitative capillary electrophoretic analysis can be achieved through internal standardization and normalization of peak areas with respect to migration times [21].

A potential disadvantage of capillary electrophoretic analysis is relatively short column lifetimes. To overcome that limitation entangled acrylamide polymer solution was developed to refill the capillary intermittently as needed during a series of analyses. Resolution was comparable to that for cross-linked capillary gel columns. Refillable capillary columns were especially useful for analysis of free oligonucleotides in liposome samples, as liposome particles can accumulate at the capillary inlet over several injections, reducing lifetime for cross-linked columns to a few runs. The replacement of the linear polyacrylamide solution can be easily achieved in every third run by inserting one end of the capillary column into the linear polyacrylamide solution in a vial and applying a pressure of 20 psi to the vial for 2 min. Therefore, analysis can continue for long periods without manual interruption.

Capillary electrophoretic resolution of oligonucleotides is dependent on four major factors: (1) electrokinetic injection; (2) detection volume; (3) diffusion; and (4) thermal gradients [20]. The first two factors are relatively fixed experimental parameters, while diffusion and thermal gradients are time and temperature (electric field) dependent, respectively. At a lower electric field, peak broadening is dominated by diffusion, while at high electric field, resolution is mainly limited by thermal gradients generated by Joule heating. Different electric fields were tested for the separation of same-sequence oligonucleotides varying in length by 1, 2 and 3 nucleotides. Fig. 2 shows a typical electropherogram of an antisense oligonucleotide drug spiked with authentic samples of synthesis deletion sequences of length $n-1$, $n-2$ and $n-3$. Resolutions between adjacent peaks at different electric fields are listed in Table 1. Best resolution was achieved at an electric field value of 600 V cm^{-1}.

Due to competitive migration among sample anions during electrokinetic injection, non-analyte salt concentration of the sample affects the amount of oligonucleotide applied to the capillary. Internal standardization solves this problem [21]. Fig. 3 shows the decrease in analyte and internal standard peak areas as a function of increasing non-analyte salt concentration in samples. Normalized peak areas remained constant, however, allowing accurate quantification of oligonucleotides in sample solutions. Internal

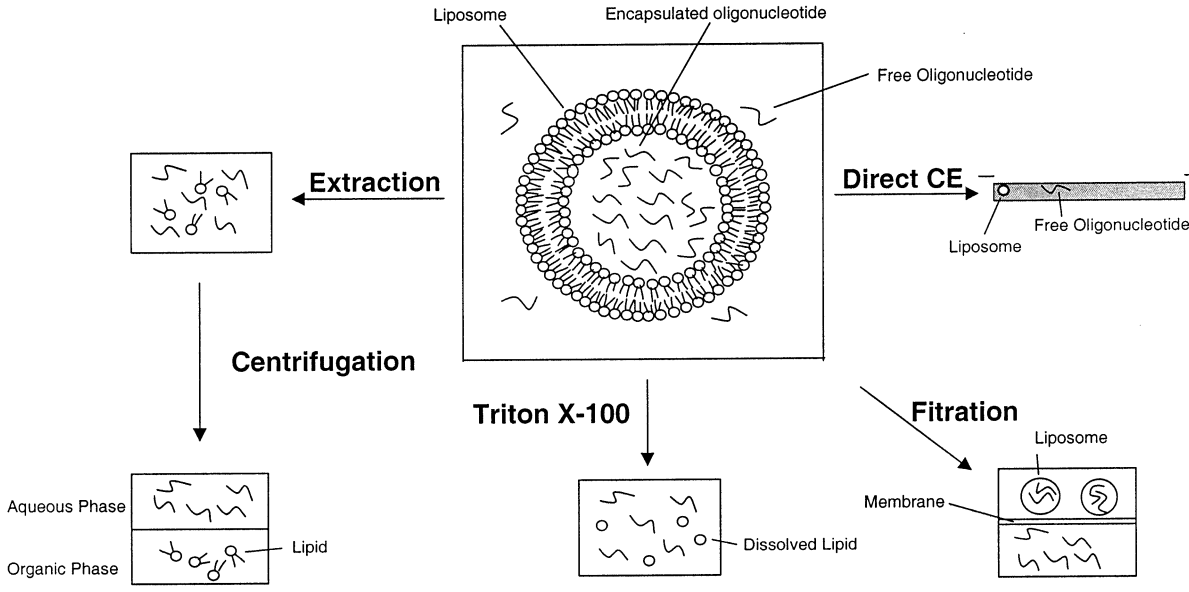

Fig. 1. A diagram for the analysis of free and total oligonucleotide in liposomes.

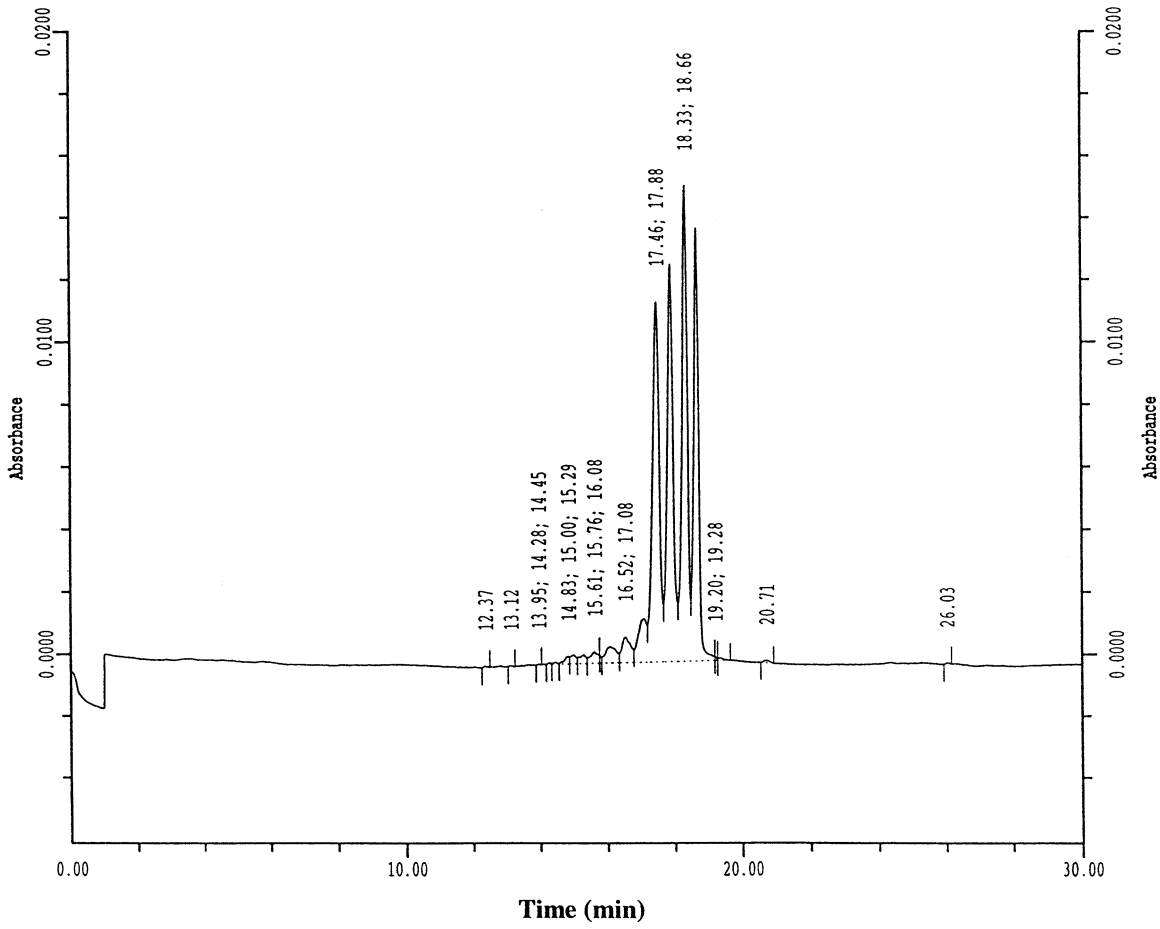

Fig. 2. An electropherogram of the oligonucleotide active pharmaceutical ingredient and the spiked authentic $(n-1)$, $(n-2)$ and $(n-3)$ deletion sequences.

Table 1
Resolutions at different electric field

Electric field (V cm^{-1})	200	300	400	500	600	700	800
$R_{n/(n-1)}$	1.08	1.09	1.18	1.15	1.30	1.29	1.17
$R_{(n-1)/(n-2)}$	1.28	1.32	1.34	1.43	1.50	1.49	1.50
$R_{(n-2)/(n-3)}$	1.09	1.10	1.16	1.24	1.32	1.26	1.24
R_{Average}	1.15	1.17	1.23	1.27	1.37	1.35	1.30

standard use also minimizes errors arising in sample preparation. The peak areas of sample and internal standard will both change proportionally if the sample solution was inappropriately diluted, therefore, the accurate analytical results can still be obtained. As shown in Fig. 4, 2-fold sample dilution (B) produced the expected reduced peak area relative to an undiluted sample (A), but both electropherograms gave the same normalized peak areas.

3.2. Determination of the total oligonucleotide

For the determination of the total concentration of oligonucleotides the first step is to disrupt the liposome structure and release the oligonucleotides, this can be achieved by phenol:chloroform extraction or using detergent.

3.2.1. Extraction

The purpose of the phenol:chloroform extraction is to remove the liposomal components away from the oligonucleotides. The lipids are extracted into the organic phase while the oligonucleotides stay in the aqueous phase. After phase separation, the aqueous solution is analyzed by capillary elec-

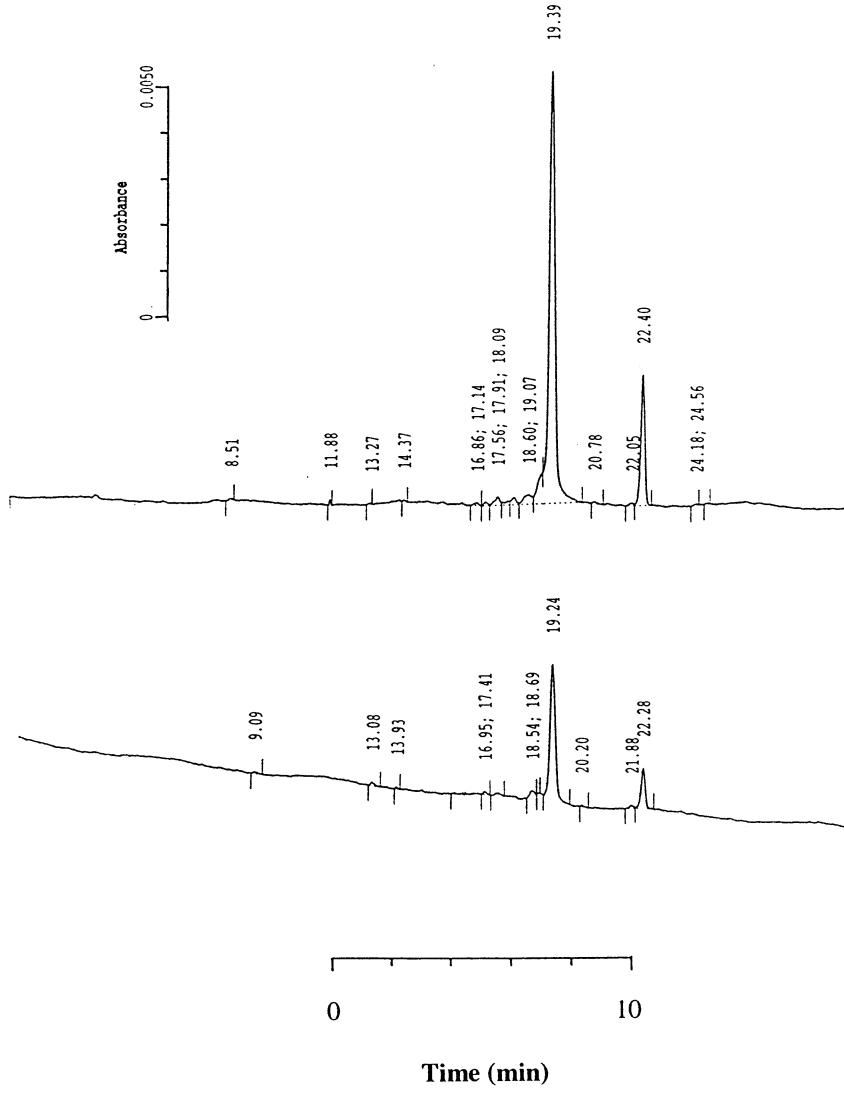

Fig. 3. The importance of internal standard in minimizing the effect of ionic strength to the accuracy. Top: Analysis of sample solution in the buffer of 0.066 M NaH_2PO_4/Na_2HPO_4 and 0.075 M NaCl (pH 7.4). Bottom: Analysis of sample solution with 2-fold of the buffer solution.

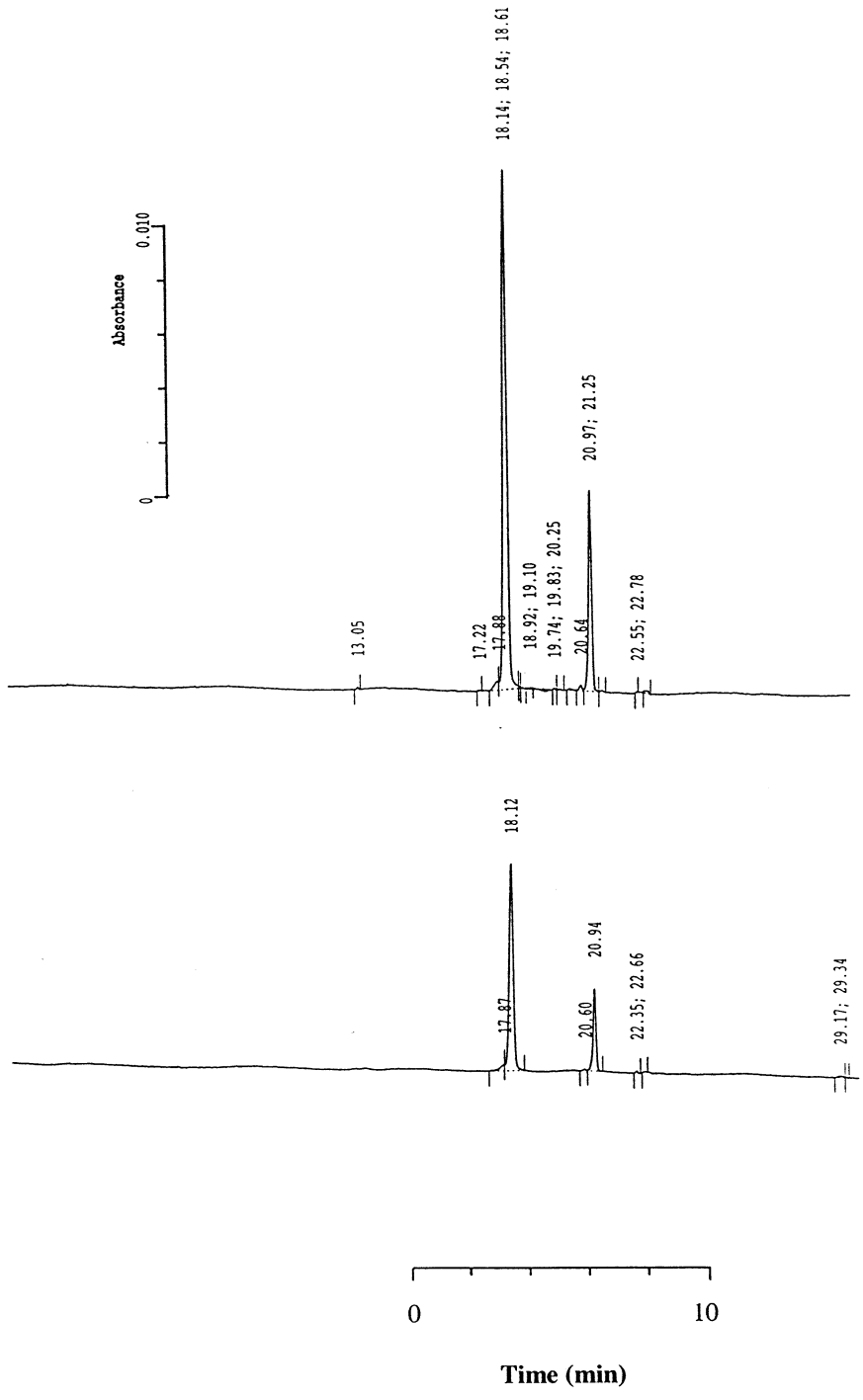

Fig. 4. The importance of internal standard in minimizing the effect of dilution error to the accuracy. Top: analysis of sample solution with normal dilution. Bottom: analysis of sample solution with 2-fold of the normal dilution.

Table 2
The recovery of oligonucleotides in the liposome sample after extraction

Without extraction		Extraction 1 (diluted with buffer)			Extract 2 (diluted with water)		
CPM	Average	CPM	Average	Recovery (%)	CPM	Average	Recovery (%)
5558.18		5466.03			5504.03		
5478.17	5520.52	5632.08	5550.08	100.5 ± 1.5	5490.08	5511.74	99.8 ± 0.5
5525.22		5552.13			5541.12		

trophoresis. Recovery of oligonucleotides was assessed by analysis of liposome samples containing known amounts of ^{35}S-labeled oligonucleotide. The liposome sample was diluted 10-fold with 5 mM histidine and 10% sucrose buffer solution and 0.5 ml of the diluted solution was extracted in triplicate. After extraction, 10 ml of the aqueous solution was added to 4 ml of scintillant solution for liquid scintillation counting. As a control, the same quantity of diluted liposome solution without extraction was added directly to 4 ml of scintillant and measured by LSC to determine recovery. To investigate the influence of buffer solution on the extraction process, liposomes containing [^{35}S]oligonucleotide were diluted with distilled water, extracted and subjected to the same LSC determination. The results are shown in Table 2. Recovery of oligonucleotides is close to 100%.

3.2.2. Detergent workup

Triton X-100, sodium dodecyl sulphate (SDS), and Tween 20 were tested for ability to disrupt liposome structure. Measured oligonucleotide concentrations increase with increasing concentration of Tween 20 in the range of 0.1–1%, and remained constant in the range of 1–10%. However, even in the 1–10% concentration range, recovery of oligonucleotides was much less than observed when using SDS or Triton X-100. SDS proved to be an efficient surfactant for disruption of liposomes. SDS charge increased the time required for electrokinetic sample injection in capillary electrophoresis analysis, however. Constant oligonucleotide concentration results were obtained for a liposome sample disrupted with Triton X-100 in the 0.1–20% concentration range. For preparation of CGE samples, liposome solution and internal standard were added to an appropriate volumetric flask and diluted to the mark with 3% Triton X-100.

Liposome samples containing different oligonucleotide concentrations were analyzed by CGE after both extraction and Triton X-100 disruption workups. The results are shown in Fig. 5. Concentrations determined by the two sample preparation methods are almost identical when the oligonucleotide concentration in liposomes is below 4 mg ml^{-1}. Slightly lower results were obtained using Triton X-100 at formulated oligonucleotide concentrations 5 mg ml^{-1}, perhaps due to insufficient disruption by the detergent. The linear regression equation in the overall range is: $Y = 0.00586 + 1.03X$ ($r^2 = 0.999$), indicating that the two sample

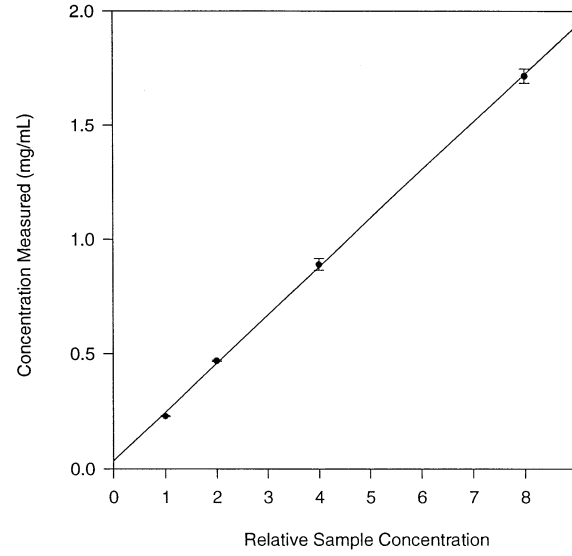

Fig. 5. Analysis of the total oligonucleotides with different dilutions after extraction.

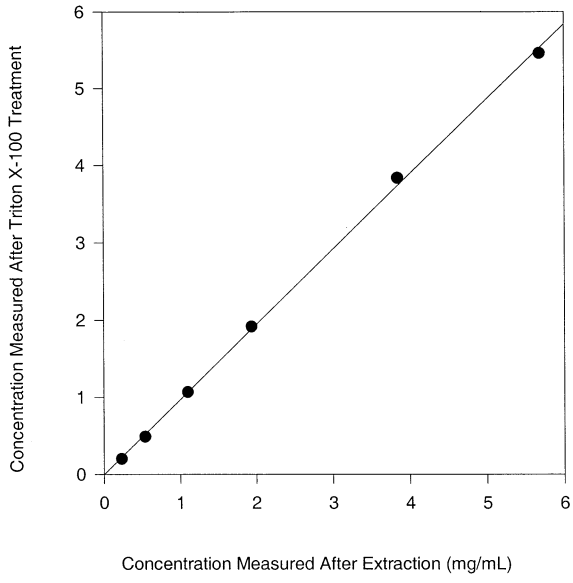

Fig. 6. Correlation between the two methods of sample treatment: extraction and Triton X-100 disruption.

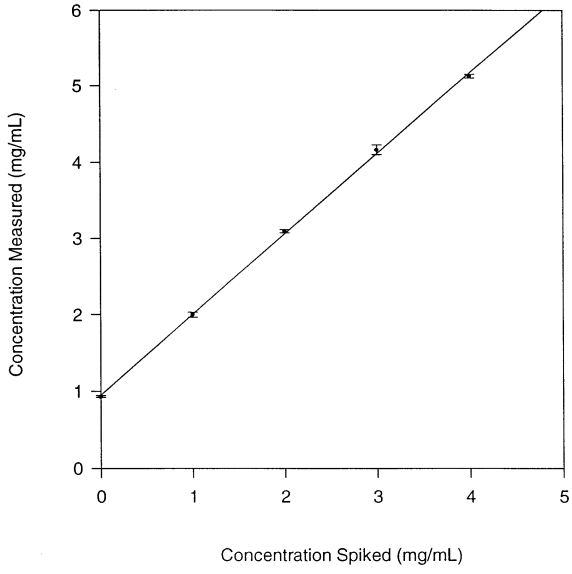

Fig. 7. Analysis of total oligonucleotides by standard addition method.

preparation methods agree to each other within 3% (Fig. 6). For best accuracy, it is recommended that the liposome first be diluted to an oligonucleotide concentration near 1 mg ml^{-1} before applying the Triton X-100 disruption procedure.

Total oligonucleotide concentration in the liposome formulations was determined against an oligonucleotide reference standard solution formulated in phosphate buffer. To verify accuracy, the oligonucleotide in liposomes was also determined using standard addition method (Fig. 7). The results obtained using both methods agree closely. Nine liposome samples were analyzed for the total oligonucleotide concentration and the results are listed in Table 3.

3.3. Determination of the free oligonucleotide

For determination of free oligonucleotide in liposome formulations, a measured volume of internal standard solution (a thymidine 27-mer oligonucleotide) was added to 1 ml of liposome sample and analyzed by capillary electrophoresis. Different quantities of oligonucleotide analyte were added to the liposome sample solutions and the free oligonucleotide concentrations determined by capillary electrophoresis. Results listed in Table 4 show that recoveries of free oligonucleotide are in the range 96.5–105.1%.

To asses possible interference of liposomes or their components in determination of free oligonucleotide, liposome solutions were added to Centricon-100 tubes (100 000 MW cutoff) for ultrafiltration prior to capillary electrophoresis. Liposome particles were in the size range 100–200 nm, therefore remained in the upper tube along with encapsulated oligonucleotides. The aqueous

Table 3
Total oligonucleotide concentration measured

Sample no.	Total oligonucleotide concentration (mg ml^{-1})
No. 1	6.95 ± 0.37
No. 2	6.61 ± 0.40
No. 3	5.47 ± 0.04
No. 4	1.63 ± 0.03
No. 5	0.94 ± 0.005
No. 6	4.27 ± 0.01
No. 7	0.39 ± 0.004
No. 8	2.89 ± 0.01

Table 4
The recovery for the determination of free oligonucleotide in liposome

Added (μg ml^{-1})	Measured (μg ml^{-1})	Average (μg ml^{-1})	S.D.	Recovery (%)
	3.39			
0	3.67	3.44	0.20	
	3.28			
	13.64			
10.4	12.96	13.36	0.35	96.5
	13.47			
	23.23			
20.8	24.65	24.23	0.87	99.9
	24.79			
	40.02			
36.4	43.91	41.87	1.95	105.1
	41.67			

Table 5
The free and encapsulated oligonucleotide concentrations obtained for the liposome samples

Sample no.	Free oligonucleotide (mg ml^{-1})	Encapsulated oligonucleotide (mg ml^{-1})	Free oligonucleotide in liposome (%)
No. 1	0.357	6.59	5.14
No. 2	0.0635	6.55	0.96
No. 3	0.308	5.16	5.63
No. 4	0.032	1.60	1.96
No. 5	0.051	0.89	5.43
No. 6	0.064	4.21	1.50
No. 7	0.006	0.38	1.54
No. 8	0.154	2.74	5.33

filtrates were analyzed for free oligonucleotide by the capillary electrophoresis. The determined concentration, 32.3 ± 0.5 mg ml^{-1}, is in good agreement with that from direct analysis (32.3 ± 0.3 mg ml^{-1}) and the standard addition method (31.9 mg ml^{-1}). This shows that direct capillary electrophoresis is reliable for determination of free oligonucleotides and that liposomes do not interfere with the analysis. Based on results from free and total oligonucleotide analyses, concentrations of encapsulated oligonucleotide and percent free oligonucleotide in liposome samples were calculated (Table 5).

4. Conclusion

Capillary electrophoresis has been demonstrated to be a simple and reliable method for the analysis of oligonucleotides in liposome formulations. Encapsulated oligonucleotides were released for analysis by treatment with 3% Triton X-100 or by phenol:chloroform extraction, permitting determination of total oligonucleotides. Free oligonucleotide concentrations were determined by direct application of the sample to the capillary column. Due to the zero net charge within the liposomes and their relatively large size, only free oligonucleotides migrate toward the anode and are detected. Method accuracy and precision were improved by use of internal standardization. Results are in good agreement with those by standard addition method. This capillary electrophoresis method can be used for routine determination of free and encapsulated oligonucleotides in liposome formulations.

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