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Quantitative capillary gel electrophoresis assay of phosphorothioate oligonucleotides in pharmaceutical formulations

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Abstract

Quantitative capillary gel electrophoresis (QCGE) has been developed for the accurate quantitation of a 21-mer phosphorothioate oligonucleotide, ISIS 2922, and its degradation products in an intravitreal formulation. The electrokinetic mode of injection employed by CGE necessitates formulation of the external reference standard in a sample matrix similar to that of the drug product and the use of an internal standard for improved accuracy and precision. The analytical method detailed in this paper has demonstrated the necessary accuracy, precision, linearity, range, selectivity and ruggedness for use in routine drug product analysis and stability monitoring of phosphorothioate oligonucleotides.

1. Introduction

Recent developments in antisense technology have resulted in the identification and development of unique classes of oligonucleotides exhibiting a diverse array of potential therapeutic applications. The natural phosphodiester oligonucleotides are rapidly degraded by serum nucleases, a problem which has been successfully overcome by incorporation of a sulfur atom in place of a non-bridging oxygen in the phosphate backbone (Fig. 1) [1]. A number of these nuclease-resistant phosphorothioates are now in active clinical development, necessitating a simple and reliable method for their quantitation in pharmaceutical preparations. Capillary gel electrophoresis (CGE) has recently gained popularity as a viable alternative to polyacrylamide gel electrophoresis (PAGE) for the analysis of synthetic oligonucleotides [2–8]. While selectivity appears to be similar to that of PAGE, CGE analysis has a number of advantages over traditional slab gel techniques, including superior resolution, on line detection, and automation. Theoretical efficiencies of over 10^7 plates/m have been reported for single-stranded oligonucleotides on capillary gel columns using cross-linked polyacrylamide [6]. Because CGE is a relatively new technique, the analytical requirements regarding its acceptability for routine drug product analysis [9] are yet to be reported in the pharmaceutical analytical literature.

Impurity profiling and drug concentration assay are the major goals in quantitative analysis

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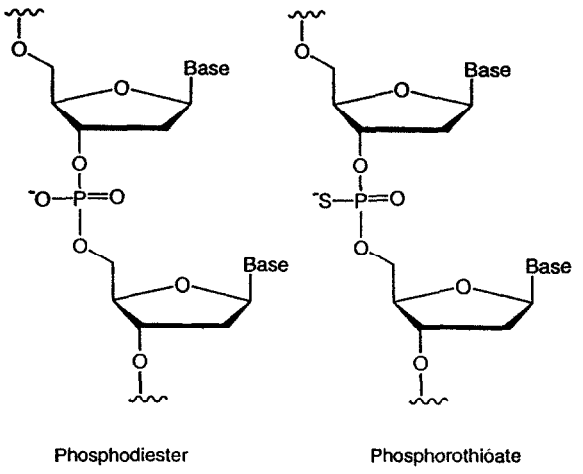

Fig. 1. Representative structures of a phosphodiester and a phosphorothioate backbone.

of drug products. Impurity profiling, the quantitation of synthesis-related deletion sequence impurities [10] is straightforward since it involves a relative area% determination. As such, CGE results are independent of applied sample concentration, so long as the concentration of the injected analyte is within the optimum range for electrophoretic resolution. Assay of drug concentration, however, is a mass/mass measurement requiring assay of the drug product sample against a reference standard of known concentration/purity. This poses a challenge for quantitative CGE (QCGE) analysis as it employs, by necessity, an electrokinetic mode of introducing the sample onto the capillary column. This places stringent requirements on the sample matrix since the quantity of analyte loaded onto the capillary is a function of the electrophoretic mobilities of the solutes present in the sample. For oligonucleotide drug formulations, buffer salts will be preferentially loaded onto the gel capillary due to their relatively high charge-to-mass ratios. Thus, relatively small differences in the amounts of buffer salts present in the sample and the external standard can lead to dramatic differences in the amount of oligonucleotide loaded onto the column and the observed detector response [11]. Most pharmaceutical products intended for intravenous or ophthalmic use are formulated in isotonic salt solutions. It is crucial to the direct, accurate quantitation of oligonucleotide concentrations in pharmaceutical dosage forms, therefore, to formulate the external reference standard in an identical sample matrix to that of the drug product being assayed. In addition, use of an internal standard has been shown to be necessary to correct for migration time and peak area irreproducibility [10]. In this paper, we present validation data to support the use of this sample preparation and dilution protocol for accurate concentration assay of a 21-mer phosphorothioate, ISIS 2922, and its degradation products in an intravitreal formulation. This approach, referred to as QCGE, has been demonstrated to have the linearity, accuracy, selectivity, precision and ruggedness required for routine drug product analysis.

2. Experimental

2.1. Chemicals and reagents

Phosphorothioate oligonucleotide, ISIS 2922, was synthesized on a solid-phase DNA synthesizer and purified by preparative reversed-phase chromatography. The drug substance was further purified by preparative strong anion-exchange chromatography, desalted by reversed-phase absorption/elution, rotoevaporated and depyrogenated prior to lyophilization. ISIS 2922 was formulated in a 0.02 *M* sodium carbonate–hydrogencarbonate buffer containing 0.13 *M* sodium chloride at pH 8.7 and the osmolality adjusted to 300 mOsm/kg [9]. Phosphorothioate analogues ($n-1$ deletion sequences and the phosphodiesters) were similarly synthesized, but ethanol precipitated. All samples were stored at 4°C and warmed to room temperature prior to analysis.

High-purity water (Barnstead Nanopure, Newton, MA, USA) was used for the preparation of samples and dilution matrices. Buffers were composed of sodium carbonate (USP grade) purchased from J.T. Baker (Phillipsburg, NJ, USA), sodium hydrogencarbonate (USP/NF grade) purchased from Spectrum Chemical Manufacturing (Gardena, CA, USA) and ACS-

grade sodium chloride purchased from Aldrich (Milwaukee, WI, USA). Pore size filters (0.2 μm) were purchased from Schleicher and Schuell (Keene, NH, USA).

2.2. Sample preparation for QCGE assay

ISIS 2922 formulations greater than 1 mg/ml were diluted to 1 mg/ml with placebo. ISIS 2922 formulations less than 1 mg/ml were diluted to approximately 0.33 mg/ml with placebo. The external standard was formulated at 1 mg/ml (or 0.33 mg/ml) on an anhydrous basis (moisture content determined by capillary gas chromatography with thermal conductivity detection) in the same placebo solution. An aliquot of internal standard, a phosphorothioate thymidine oligonucleotide 23-mer (T_{23}), was added to the standard and samples and both were diluted to an analyte range of 0.33 to 10 μg/ml with deionized water to minimize the salt concentration prior to CGE analysis. The final concentration of the internal standard was in the range of 0.75 to 2.5 μg/ml.

2.3. Capillary gel electrophoresis apparatus

Electrophoretic data were acquired using a Beckman (Fullerton, CA, USA) 2000 or 5000 P/ACE instrument operated at an applied voltage of 14.1 kV. The column temperature was maintained at 30°C and detection was at 254 or 265 nm. 47 cm polyacrylamide gel columns U100P (catalog No. 338480) with an effective column length of 40 cm (7 cm from the detector to the waste reservoir) and the running buffer Tris–Borate/Urea (catalog No. 338481) were purchased from Beckman. Electrokinetic injections were made at an injection voltage of 7 kV for 30 and 20 s for 0.33 and 10 mg/ml samples, respectively. Column lifetimes were in the order of 50 to 75 ISIS 2922 drug product samples.

2.4. Calculations

In QCGE analysis, the peak areas are influenced by the migration velocities of the analytes leading to differences in the residence time at the detector. This may be corrected for by simply dividing the observed peak area by the corresponding migration time and is termed "corrected peak area". To compensate for differences in sample loading between injections, the "corrected peak area" is further normalized to the detector response of the internal standard, T_{23}, and is then termed "normalized peak area". The "normalized migration time" is calculated by dividing the migration time of the analyte by that of the internal standard.

3. Results and discussion

3.1. Precision in CGE

A number of experimental parameters have a significant impact on the migration time and the detector response of the analyte signals in CGE. These include temperature, sample matrix effects and electrokinetic injection effects. These factors may be dealt with adequately by use of a suitable internal standard in combination with strict control of the experimental conditions. T_{23} was chosen as the internal standard for the CGE analysis of ISIS 2922.

3.2. Migration time reproducibility: effect of an internal standard

Table 1 summarizes the migration time data for a series of ISIS 2922 drug product samples run on a single day. Also shown are the normalized migration time of ISIS 2922 (dividing the migration time of ISIS 2922 by that of T_{23}). Normalizing the observed migration times effec-

Table 1
Migration time reproducibility ($n = 12$)

	Observed	Normalized to T_{23}
Mean migration time (min)	30.085	0.9729
S.D. (min)	0.419	0.0006
R.S.D. (%)	1.39	0.06

tively compensates for migration differences between sample runs, resulting in a significant improvement in the R.S.D. from 1.39 to 0.06%.

3.3. Area% reproducibility: effect of an internal standard

Table 2 shows reproducibility data for a series of ISIS 2922 injections using corrected peak areas and the same data when normalized to the T_{23} internal standard. A known amount of T_{23} was added to each of the nine samples of ISIS 2922 prior to analysis. The effect of the internal standard is shown by the dramatic improvement in the R.S.D. of the integrated area of ISIS 2922 from 46.80 to 1.75%. Similarly, the R.S.D. for the quantitation of $n-1$ deletion sequences improves from 41.73 to 5.94%.

3.4. Electrokinetic injection effects

Electrokinetic injection in CGE is performed by replacing the injection-end reservoir with the sample vial and applying a voltage 2 to 5 times lower than that used for the separation. In this form of injection, the analyte enters the capillary primarily by migration and by the effects of electroosmotic flow. The length of electrokinetic injection time and the applied voltage directly impact the amount of analyte introduced onto the gel capillary column, i.e., the longer the injection time and/or the higher the applied voltage, the more analyte loaded onto the column. The quantities loaded are also a function of the electrophoretic mobilities of individual analytes. For ISIS 2922 and its related $(n-1)$ sequences, there is little to no discrimination in sample loading as a function of length because their charge-to-mass ratios are essentially the same. However, any buffer ions present in the sample matrix would be preferentially loaded due to their relatively high charge-to-mass ratios. In summary, efficiency and reproducibility of the electrokinetic sample injection of ISIS 2922 require adequate control of the length of the electrokinetic injection, applied voltage during injection and the sample matrix. These factors are addressed in detail below.

3.5. Effect of electrokinetic injection time

Table 3 demonstrates the effect of injection time on the observed corrected peak area of a sample of ISIS 2922 in Tris–borate/7 *M* urea running buffer. The corrected peak area in-

Table 2
Reproducibility of integrated peak area

	Corrected peak area		Normalized to T_{23}	
	ISIS 2922	$n-1$	ISIS 2922	$n-1$
	0.168487	0.01072	5.61391	0.35184
	0.111345	0.00714	5.47222	0.34452
	0.154746	0.01114	5.83005	0.41076
	0.162939	0.01040	5.73130	0.35786
	0.196860	0.01257	5.71057	0.35675
	0.115623	0.00722	5.70747	0.33618
	0.104393	0.00650	5.74548	0.35177
	0.281972	0.01698	5.71057	0.35675
	0.362852	0.02071	5.70931	0.34879
Mean	0.1844	0.0122	5.6923	0.3572
S.D.	0.0863	0.0051	0.0994	0.0212
R.S.D. (%)	46.80	41.73	1.75	5.94

Table 3
Influence of electrokinetic injection time ($n = 3$) on peak area

Injection time (s)	Corrected peak area		Area%	
	ISIS 2922	$n-1$	ISIS 2922	$n-1$
5	0.0084 ± 0.0001	0.00040 ± 0.00004	95.68	4.32
10	0.0132 ± 0.0007[a]	0.00067 ± 0.00009	95.13	4.87
20	0.0291 ± 0.0004	0.00133 ± 0.00011	95.64	4.36
		Mean	95.48	4.52
		S.D.	0.31	0.31
		R.S.D. (%)	0.32	6.8

Sample: 0.24 mg/ml ISIS 2922 in Tris–borate buffer with 7 *M* urea. Electrokinetic injection at 7 kV for 5, 10 and 20 s.
[a] $n = 2$.

creases as a function of electrokinetic injection time. Note that there is no bias in the loaded quantity of ISIS 2922 relative to its $n-1$ deletion sequence as a function of injection time. This is shown by the lack of an increasing or decreasing trend in the area% data for ISIS 2922 relative to its $n-1$ deletion sequence. The higher R.S.D. associated with $n-1$ quantitation reflects the error due to integration of lower detector signals. For routine quantitative analysis of ISIS 2922, the electrokinetic injection time should be well defined and controlled.

3.6. Effect of sample matrix

Presence of buffer salts in the sample results in preferential loading of these salts onto the capillary column resulting in reduced and irreproducible peak areas for the analyte of interest. An extreme case of this effect is demonstrated by the data in Table 4 showing a 0.24 mg/ml sample of ISIS 2922 dissolved in deionized water and in the 7 *M* Tris–borate/urea running buffer. The corrected peak areas for ISIS 2922 are significantly higher when dissolved in water than in the Tris/urea buffer.

Table 4
Influence of sample matrix on corrected peak area of ISIS 2922

Sample matrix	Corrected peak area
Deionized water (4.4 S)	0.1792 ± 0.045
Tris–borate/7 *M* urea (440.0 S)	0.0084 ± 0.0001

7 kV, 5-s injection, $n = 3$.

Although this is not a problem for the analysis of ISIS 2922 bulk drug substance, a lyophilized powder which may be readily dissolved in deionized water prior to analysis, it does pose a challenge for the analysis of ISIS 2922 drug product. ISIS 2922 (as with most pharmaceutical preparations) is formulated in a buffer solution and the osmolality adjusted to 300 mOsm/kg to render it suitable for intravitreal dosing. Direct analysis of the drug product solution requires formulation of the ISIS 2922 reference standard in placebo such that the sample matrices are identical. Sample preparation and dilution protocols for the assay of ISIS 2922 Intravitreal drug product are discussed in detail in the Experimental section.

3.7. Limit of detection

The limit of detection for a 5 mg/ml ISIS 2922 intravitreal drug product (following the 1:100 dilution scheme with deionized water described in the Experimental section) was determined to be 0.084 μg/ml as analyzed on a P/ACE 2000 instrument or as low as 0.05 μg/ml when measured on a P/ACE 5000 which is equipped with a higher quality optic system.

3.8. Linear dynamic range

Linearity of the detector response (mAU) was demonstrated in the range of 0.05–12.5 μg/ml ($y = 0.437 \pm 0.007x - 0.014 \pm 0.374$, $r = 0.9999$) for ISIS 2922 drug product samples upon a 100-fold dilution with deionized water. Working concentrations of ISIS 2922 drug product samples following the same dilution protocol are typically between 0.33–10 μg/ml falling well within this linear dynamic range.

3.9. Selectivity

CGE is primarily a length-based separation yielding excellent resolution of the deletion sequences of ISIS 2922 (Fig. 2). However, "thiation failures" (mono or higher order partial phosphodiesters) are undetected by CGE (Fig. 3). An electropherogram of ISIS 2922 and an $(n-1)$ deletion sequences of ISIS 2922 are shown in Fig. 2a and b, respectively. Fig. 2c shows the co-injection of the full-length ISIS 2922 and the $n-1$ deletion sequences demonstrating length-based resolution of CGE. Fig. 3 demonstrates the comigration of ISIS 2922 and its monophosphodiester analogue. All higher-order phosphodiesters including the total phosphodiester sequence of ISIS 2922 were found to comigrate with ISIS 2922. In summary, CGE analysis is inherently a measure of the amount of full-length ISIS 2922 in a given sample and does not detect nor distinguish the replacement of a (single or multiple) sulfur atom by an oxygen in the phosphate backbone. The presence of these related oligonucleotides, if present, can be independently detected and quantitated by anion-exchange chromatography or phosphorous NMR.

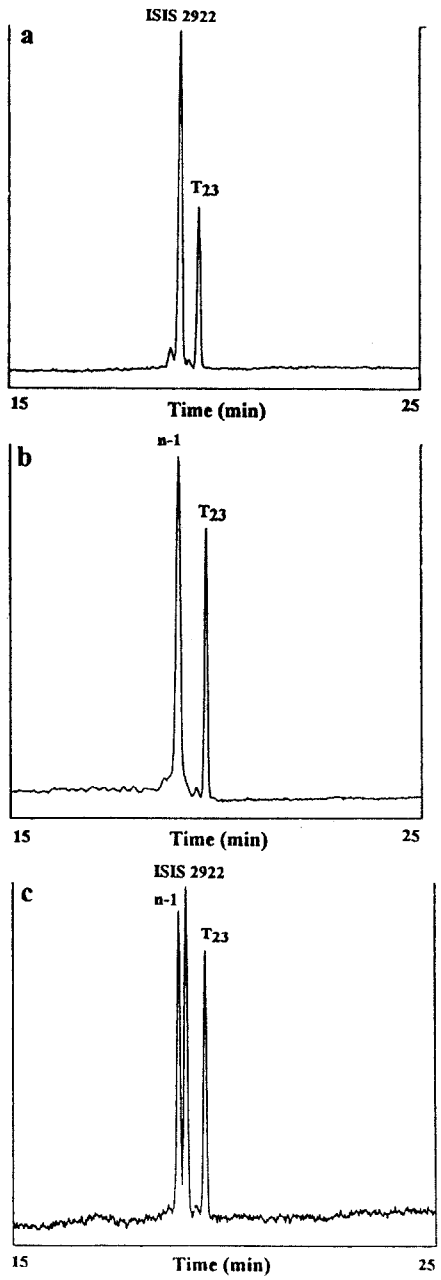

Fig. 2. Capillary gel electropherograms of (a) ISIS 2922, (b) an $n-1$ deletion sequence of ISIS 2922 and (c) a mixture of (a) and (b) demonstrating length-based resolution. Detection by UV absorbance at 254 nm.

3.10. Ruggedness

Day-to-day

The area% reproducibility of an ISIS 2922 solution from day-to-day using the same capillary gel column is 0.43% R.S.D., and precision of the normalized migration time is on the order of 0.051% R.S.D. These data are summarized in Table 5.

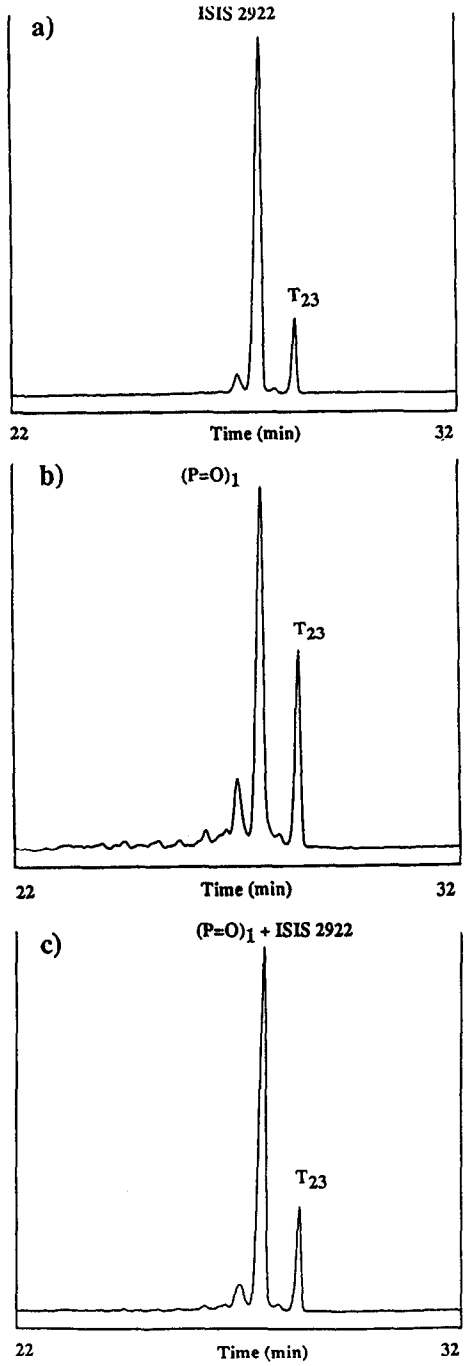

Fig. 3. Capillary gel electropherograms of (a) ISIS 2922, (b) a monophosphodiester of ISIS 2922 and (c) a mixture of (a) and (b) demonstrating lack of resolution when a sulfur is replaced by an oxygen in the oligonucleotide backbone. Detection by UV absorbance at 254 nm.

Column-to-column

CGE analysis of ISIS 2922 uses commercially available gel-filled capillaries with a column lifetime in the range of 50 to 75 injections of ISIS 2922 drug product samples. For this reason, it is important to evaluate the variability of the observed peak areas between different columns. Column-to-column reproducibility was assessed across a two-month period using the same 6 mg/ml ISIS 2922 drug product sample which was stored at 4°C during the course of the study. The results of this study are presented in Table 6. As shown, the relative precisions for mean normalized migration time and mean area % were found to be 0.092 and 0.440%, respectively, between columns. Additional ruggedness data for ISIS 2922 drug product assay is shown in Table 9.

3.11. Accuracy of the QCGE assay of ISIS 2922 drug product

Accuracy (recovery) of the QCGE assay was determined by assaying ISIS 2922 that was formulated in pH 8.7 sodium carbonate–hydrogencarbonate buffer at 1.0 and 0.33 mg/ml on an anhydrous basis. Moisture content of the bulk drug substance was determined immediately prior to formulation. This sample was assayed against a primary reference standard of known purity. The results summarized in Table 7 show that recovery of the CGE assay at both 0.33 and 1.0 mg/ml is nearly 100%.

3.12. Precision of the QCGE assay of ISIS 2922 drug product

Overall reproducibility of the QCGE assay of ISIS 2922 drug product was evaluated by analyzing three lots of drug products against formulated reference standards (in triplicate). Because these measurements are against triplicate analyses of the formulated reference standard (three individual weighings followed by formulation and subsequent dilutions; includes separate moisture determinations of the reference standard at the time of analysis), the data presented in Table 8 represent the overall precision of the QCGE assay as it would be performed during

Table 5
Day-to-day precision of CGE analysis of ISIS 2922

Day	Mean area	Mean area %	Mean normalized migration time	*n*
1	6.905	95.00	0.9732	3
2	6.871	94.65	0.9722	3
3	6.961	95.47	0.9724	3
Mean	6.912 ± 0.05	95.04 ± 0.41	0.9726 ± 0.0005	9
R.S.D. (%)	0.66	0.43	0.051	

Table 6
Column-to-column reproducibility of CGE analysis of ISIS 2922

Column	Mean area	Mean area %	Mean normalized migration time	*n*
1	6.234	94.55	0.975	8
2	6.392	95.07	0.973	8
3	6.171	94.77	0.974	8
Mean	6.234	94.80	0.974	24
S.D.	0.109	0.417	0.001	
R.S.D. (%)	1.735	0.440	0.092	

routine analysis. R.S.D.s ranged between 1.1 and 3.2% for the analysis of 1.0 and 0.33 mg/ml ISIS 2922 drug product samples. Drug product concentrations higher than 1 mg/ml would be diluted to 1.0 mg/ml prior to analysis. Mean assay values reported in Table 8 range between 100 and 106% label claim for the three lots. In another study, two ISIS 2922 drug product samples were assayed four times over a six-week time period. The data are presented in Table 9. Precision of the 0.33 mg/ml sample and 5 mg/ml at 2.75 and 2.85%, respectively, should reflect the long-term assay variance that would be observed for a stability study.

Table 7
Accuracy of the QCGE assay of ISIS 2922 drug product

Theoretical	Experimental	
1 mg/ml	Mean (mg/ml)	1.008 ± 0.022
	% of theoretical	100.8
	R.S.D. (%)	2.18
	n	10
0.33 mg/ml	Mean (mg/ml)	0.331 ± 0.010
	% of theoretical	100.3
	R.S.D. (%)	3.05
	n	9

4. Conclusions

CGE is rapidly gaining popularity for the separation of oligonucleotides. In this paper, we demonstrate its validity as a quantitative technique for the determination of ISIS 2922 concentration in a pharmaceutical formulation by systematic evaluation and control of the experimental parameters that impact quantitative analysis. The importance of formulating the external reference standard in an identical sample matrix as that of the sample to overcome the inherently poor injection to injection reproducibility of electrokinetic injections has been demonstrated for accurate drug product assay. The utility of using an internal standard to correct for

Table 8
Precision of the QCGE assay of ISIS 2922 drug product

	Label claim (mg/ml)	Lot No. A	Lot No. B	Lot No. C
% Label claim		104.0	104.8	99.6
Mean concentration	1	1.04	1.053	1.00
S.D.		0.023	0.034	0.011
R.S.D. (%)		2.21	3.24	1.10
n		4	6	7
% Label claim		99.7	105.7	102.4
Mean concentration	0.33	0.329	0.349	0.338
S.D.		0.008	0.011	0.006
R.S.D. (%)		2.43	3.15	1.78
n		2	5	4

inherent migration time and peak area irreproducibility has also been shown. In summary, QCGE has been demonstrated to be an acceptable technique for the routine analysis of phosphorothioate oligonucleotide drug product formulations. While this paper addresses quantitation of a specific oligonucleotide-based drug, the analytical approach described has wide applicability to the assay of any formulated drug product that can suitably be separated by CGE.

Table 9
Inter-assay precision over a six-week time period of the QCGE assay of ISIS 2922 drug product

	Concentration of ISIS 2922 (mg/ml)	
Label claim	0.33	5.0
Initial	0.334	5.41
Week 2	0.357	5.50
Week 4	0.343	5.16
Week 6	0.346	5.26
Mean	0.345	5.33
S.D.	0.009	0.152
R.S.D. (%)	2.75	2.85

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