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Development and validation of a method for routine base composition analysis of phosphorothioate oligonucleotides

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Abstract: A method for routine base composition determination of phosphorothioate oligonucleotides containing as many as 21 nucleobases has been developed and systematically evaluated in terms of factors contributing to assay precision and accuracy. Phosphorothioate internucleotide linkages were oxidized with a mixture of tetrahydrofuran–water–methylimidazole (16:4:1, v/v/v) which has been shown to be 97.3% effective. This step was followed by enzymolysis and HPLC quantitation of individual nucleobases. RSD for inter-day base composition analysis ranged from 1.1 to 1.3%, and inter-lot variation was 0.6–2.0%. Accuracy of the determined nucleobase ratio was independently confirmed through sequencing of the oxidized oligomer by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

Keywords: *Base composition; phosphorothioate oligonucleotides; HPLC.*

Introduction

A critical step in drug substance control is establishing absolute identity of the drug. For synthetic oligonucleotides, this may require sequencing and nucleobase composition analysis [1]. Although this is straightforward for native phosphodiester oligonucleotides [2–11], the resistance of phosphorothioate oligonucleotide drugs to chemical and enzymatic cleavage greatly hinders these identification tests. Oxidation of the phosphorothioate linkages by reaction with iodine prior to digestion with endo- and exonucleases has been shown to facilitate enzymolysis [12–14]. A limited number of groups have investigated the base composition of short phosphorothioate oligomers; however, none of these methods were suitable for routine identification of longer (composed of ≥20 bases) phosphorothioate oligonucleotide drugs as typically characterized in our laboratory. For example, Connolly *et al.* [14] have demonstrated effective oxidation of a phosphorothioate octamer with iodine/pyridine. This approach was not adaptable to routine analysis since removal of oxidation reagents was cumbersome and residual pyridine masked or

complicated the detection of 2′-deoxyadenosine. Agrawal *et al.* [13] have claimed sodium metaperiodate to be a rapid and cleaner oxidizing agent, although thorough details of experimental conditions were not included and results for longer sequences were not reported. An earlier, alternative approach employed by Agrawal *et al.* [15] removed a portion of the synthetic oligomer from the solid synthesis support prior to addition of sulphur or amine and treated the oligomer with a 2% solution of iodine in pyridine–water (98:2, v/v) as a means of simplifying the oxidation step. However, for routine drug substance control, it is important to confirm composition of the *final* phosphorothioate drug substance. Finally, a recent report describes oxidation using a mixture of iodine in tetrahydrofuran (THF)–water–methylimidazole–(MI) (16:3:1, v/v/v) for base authentication of a di- and trinucleoside containing diphosphorothioate linkages [12]. During our evaluation of this approach, it was observed that the relative amount of water used affects efficiency of the desulphurization.

In this paper, we report a modification of the THF/H$_2$O/MI/iodine oxidation method which renders it suitable for routine base composition determination of phosphorothioate oligo-

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nucleotides containing as many as 21 bases. Among the factors considered during development and validation of this routine base composition assay were total analysis time, adaptability to routine analysis, simplicity of the experimental protocol, efficiency of the desulphurization step, ease of removing potentially interfering reagents, and robustness of the assay for determination of base composition of 20-base phosphorothioate oligonucleotides.

Experimental

Materials

Phosphodiesterase I (SVPD) (100 U) was purchased from United States Biochemicals (Cleveland, OH, USA). Nuclease P_1 (29.7 U μl^{-1}) and calf intestinal alkaline phosphatase (CIP) (1000 U; 23 U μl^{-1}) were obtained from Gibco BRL (Gaithersburg, MD, USA). Deoxynucleotide standards [2'-deoxyguanosine (99–100%), thymidine (99–100%), 2'-deoxyadenosine (99–100%), 2'-deoxyinosine (99–100%), and 2'-deoxycytidine (99–100%) were purchased from The Sigma Chemical Co. (St Louis, MO, USA). ACS reagent grade iodine (100%), pyridine (>99%), sodium *m*-periodate, and HPLC grade ether (99.9%) were also purchased from Sigma. $MgCl_2$ from Sigma, Ultra Pure™ enzyme grade Tris (Tris-(hydroxymethyl) aminomethane hydrochloride) from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD, USA) and Mallinkrodt analytical reagent grade NaCl (Paris, KY, USA) were used for the 10× buffer. Omnisolv™ grade tetrahydrofuran (THF) and acetonitrile (ACN) were obtained from EM Science (Gibbstown, NJ, USA), and >99% redistilled 1-methylimidazole was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Triethylammonium acetate (TEAA) (1.0 M) was purchased from Fluka Bio-Chemika (Ronkokoma, NY, USA). Solutions of TEAA and ACN were filtered using a 0.2-µm filter setup prior to use in chromatography. Centrifugal 0.2-µm Z-spin filtration units (Gelman) sold through Curtin Matheson Scientific, Inc. (Houston, TX, USA) were used for removing foreign particles from the sample prior to injection on the column.

Apparatus

A Hewlett–Packard 1090 Series II HPLC equipped with a 4.6 × 100 mm, Hypersil ODS

column (sold through Hewlett–Packard) was used for separation of individual nucleosides.

Preparation of samples

Direct digestions. To approximately 0.5 OD_{260} of phosphorothioate oligonucleotide, was added 2 µl of a 10× buffer (pH = 8.5) containing 50 mM Tris, 14 mM $MgCl_2$, and 72 mM NaCl. A stock solution of SVPD was prepared by adding 1 ml of a mixture containing 1:1 glycerol and 1× buffer (prepared by dilution of the 10× buffer). The stock solution was vortexed and stored at −20°C. To the phosphorothioate oligonucleotide mixture was added 2 µl SVPD, 2 µl nuclease P_1, 2 µl CIP, and enough water (pH 7.0) to bring the total volume to 20 µl. For experiments requiring consecutive periods of digestion, the total volume was increased by 20 µl with each addition of enzymes/reagents. The mixture of enzymes and phosphorothioate oligonucleotide was vortexed briefly, centrifuged, and incubated at 37°C for 16–96 h. Samples were periodically centrifuged during the incubation in order to consolidate any enzymes and oligonucleotide condensed on the inside of the cap toward the bottom of the centrifuge tube. Following the incubation, each sample was briefly centrifuged through a 0.22-µm filtered centrifuge tube. The collected contents were transferred to a 100-µl glass crimp top vial and inserted into the HPLC autosampler tray. Gradient conditions were as follows, using 0.1 M TEAA and ACN: $t = 0$–4.99 min, 0% ACN; $t = 5.0$–20.0 min, 2.0–10.0% ACN; 20.0–25.0 min, 10.0–70.0% ACN; 25.0–30.0 min, 70.0–0.0% ACN. A post equilibration period of 2.5 min was added to the end of the run. The flow rate was set at 1.0 ml min⁻¹ and a 5-µl injection volume of the sample was used.

Pyridine/iodine treatment. Seventy-five microlitres of pyridine/0.5 mg iodine was added to 0.5 OD_{260} units of oligonucleotide and allowed to react for 45 min. Following the desulphurization, the contents of the centrifuge tube were added to 1 ml of water in a glass scintillation vial and the components were vortexed for 30 s. To this was added 2 ml of anhydrous ethyl ether in order to extract the iodine and pyridine. The vial was capped and mechanically shaken for 2 min after which the ether layer was removed. The extraction procedure was repeated four more times after which the contents were transferred to a

centrifuge tube, dried using a Speed Vac and thoroughly dissolved in 200 μl of water. The solution was passed through a filtered centrifuge tube to remove residual iodine particles and the filtrate was then dried a second time. Enzymes were added to the residue over night and the mixture was again passed through a 0.2-μm filtered centrifuge tube prior to analysis to remove any enzyme particulates.

Sodium metaperiodate/water treatment. Approximately 0.8 mg of sodium metaperiodate/OD_{260} oligonucleotide was dissolved in 100 μl water. The oxidation of the phosphorothioate was allowed to proceed for 2 h. Removal of iodine was accomplished by diluting the sample with 250 μl water, vortexing and loading the entire sample onto a size exclusion Sephadex gel column. Samples were collected at 1.5 ml intervals using water treated with four drops of ammonia/l water. The phosphodiester oligonucleotide was eluted between 9 and 12 ml and was located using UV–vis measurements at 267 nm. Approximately 60% recovery of the sample was possible. Samples containing the phosphodiester were then collected and dried using a Speed Vac system. Enzymes were added directly to the residue as described for the direct digestion. Incubation proceeded for 16–24 h.

THF/water/methylimidazole/iodine treatment. Fifty microlitres of a 16:4:1 (v/v/v) THF–water–methylimidazole mixture which contained 1 mg iodine/100 μl solution was added to 0.5 OD_{260} of the phosphorothioate oligonucleotide and reacted for 2.0 h at 37°C. The sample was diluted with 200 μl water, vortexed thoroughly, and the iodine was then removed according to the same procedure as for the sodium metaperiodate treatment. RP-HPLC was used to follow the exfent of oxidation since the retention times for the full phosphodiester analogue (2.0 min) and the full phosphorothioate (8.8 min) are well-separated. Oxidation was shown to be 93.7% effective under these reaction conditions.

Preparation of standards

Fifty millilitres of a 1.0 mM stock solution of standards was prepared in triplicate by weighing the following standards into each of three 50-ml volumetric flasks: 2'-deoxyadenosine (12.56 mg), 2'-deoxycytidine (11.36 mg), 2'-deoxyguanosine (13.36 mg), 2'-deoxyinosine (12.61 mg) and thymidine (12.11 mg). The 2'-deoxyinosine was included in the standards to account for the presence of the deamination product of deoxyadenosine. The flasks were diluted to mark with HPLC grade water and mixed thoroughly for 30 min using a mechanical shaker.

A set of standards was then prepared in triplicate by accurately trnsferring 5, 10 and 20 ml of the stock solution to individual 25-ml volumetric flasks and diluting to the mark with HPLC grade water to give a set of standards having concentrations ranging between 2 and 8 × 10^{-4} M, respectively. The original stock solution was used as the fourth standard in the set having a concentration of 1.0 mM. These individual standards were mixed vigorously by vortexing for 1 min.

Calculation of base composition

Peak area vs nanomoles of standard was plotted for each standard where the peak area used for each nucleoside corresponded to the appropriate maximum wavelength for each nucleoside. Peak areas for deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine were recorded at 260, 271, 254 and 267 nm, respectively. The slope for each nucleoside linear regression was also recorded. The concentration of each nucleoside present in the sample was calculated by dividing the observed peak area for that nucleoside at the appropriate maximum wavelength by the corresponding standard linear regression slope.

As an example, the relative deoxyguanosine composition was calculated as follows:

$$\text{No. of dGs} = \frac{B}{\sum C_{dA,dC,dG,dI,T}} \times C_{dG} \quad (1)$$

where B represents the total number of bases expected in a sequence and $\sum$ indicates the summation of all calculated concentrations of nucleosides present. The calculation was repeated for each type of nucleoside present. The presence of deaminase in certain lots of snake venom phosphodiesterase caused conversion of deoxyadenosine to deoxyinosine. In these cases, the concentrations of dA and dI were added in order to determine the overall concentration of dA initially present in the sample.

Results and Discussion

During the initial stages of method development, modifications to existing base composition methods for analysis of natural phosphodiester oligomers were evaluated for increasing the efficiency of *direct* digestion of phosphorothioate oligonucleotides. A combination of enzymes which stereospecifically attack linkages from the 3' end, such as nuclease P_1 and SVPD, followed by CIP which cleaves the 5'-phosphate residue, provided a more extensive digestion of the oligonucleotide which was reportedly independent of the chirality of phosphorothioate linkages [16–19].

Direct digestion

Although the activities of nucleases are considerably hindered by incorporation of sulphur throughout the oligonucleotide backbone, complete digestion of the phosphorothioate oligonucleotide may be effected through a combination of increasing the enzyme–oligonucleotide concentration ratio and the incubation time as shown in Table 1. Optimal conditions for direct digestion appear to involve a sample concentration of 0.025 mg ml^{-1} and two consecutive additions of enzymes within a 96-h digestion period, to produce the expected ratio of 6 C: 5 G: 10 T. Nevertheless, direct digestion lacks the robustness required for routine analysis. Figure 1 depicts a chromatogram for 0.67 mg ml^{-1} of directly digested ISIS 2922. The presence of peaks corresponding to large molecular weight fragments between 10 and 20 min suggests incomplete digestion of the sample and may potentially prevent accurate quantitation of deoxyadenosine, which has a retention time of approximately 11 min.

Pyridine/iodine treatment

Oxidation with a pyridine/iodine solution has been reported to be a gentle and efficient technique in comparison with more traditional oxidation reactions [14]. Figure 2(a) demonstrates effective cleavage of a 20-base deoxyadenosine-containing phosphorothioate oligonucleotide using this method of desulphurization. It was noted that the failure to remove trace amounts of pyridine hindered the quantitation of deoxyadenosine which coelutes with pyridine (the broad, tailing band centered at ca. 11.0 min in Fig. 2a). Although manual integration of the dA shoulder peak produced a ratio consistent with the expected value for the 20-base sequence (Table 2), reproducibility would be problematic. For sequences in which deoxyadenosine is expected to be absent, such as for ISIS 2922, residual pyridine does not interfere with the calculation of base composition; however, if detection of deoxyadenosine were to be used as an indicator of potential cross contamination as a result of procedural clean-up failures or unintended incorporation of deoxyadenosine during synthesis, then excess oxidation reagents would be expected to mask the detection of residual amounts of deoxyadenosine present in the sample.

In general, this approach was found to be precise but unreliable. A range of partially digested fragments eluting between 12 and 20 min suggests that cleavage was incomplete. Figure 2(b) is an example of an inefficient enzymatic degradation of ISIS 2922. Repetition of this analysis produced a thorough digestion and composition calculations were accurate (see Table 2). Incubation times between 8 and 15 h were initially determined to be adequate for producing acceptable base ratios. However, the inability to reproduce acceptable base composition values for ISIS 2922 was noted upon extended use of the method (see Day 1 vs Day 2 data).

Sodium metaperiodate/water treatment

The use of aqueous sodium metaperiodate, as described by Agrawal *et al.* [13] for oxidation of an eight member phosphorothioate oligonucleotide, eliminates potential interference by pyridine and allows effective removal of metaperiodate by simple gel filtration. Since exact conditions were not reported for the oxidation reaction, it was first necessary to optimize the reaction for conversion of a longer phosphorothioate oligonucleotide. Table 3 summarizes the influence of the iodine–sulphur ratio on the accuracy of base ratios calculated for several phosphorothioate samples treated with sodium metaperiodate. Porritt and Reese [12] recommend that a large excess of iodine (ca. 10 mole equivalents of iodine/sulphur atom) be used to force complete desulphurization. Increasing iodine equivalents from 62 to 335 compensates for the shorter digestion period of 12 vs 16.5 h: this is supported by a significant difference in the base ratios for ISIS 2922. Increasing digestion time to 24 h does not significantly improve the

Table 1
Effect of incubation time and relative enzyme–oligonucleotide concentration on efficiency of direct digestion

Sample	Concentration* (mg ml^{-1})	Incubation time (h)	Base ratio†			
			dC	dG	T	dA
ISIS 2922:089§	0.25	24	3.5	8.7	8.8	0.0
	0.25	24 + 48‡	4.6	8.0	8.4	0.0
	0.025	48 + 48	6.1	5.1	9.8	0.0
ISIS 2922:0245	0.067	48 + 24 + 24	5.9	7.4	7.7	0.0
	0.367	48 + 24 + 24	5.4	8.6	6.9	0.0

*Concentrations of oligonucleotide expressed do not account for water content of ISIS 2922.
†The base ratio determined for the ISIS 2922 full phosphodiester homolog (6841) is 5.9 ± 0.1 dC:5.4 ± 0.1 dG:9.8 ± 0.1 T:0.0 dA.
‡The "+" sign indicates addition of enzymes following initial aliquot.
§The expected ratio for ISIS 2922 is 6 dC:5 dG:10 T:0 dA.
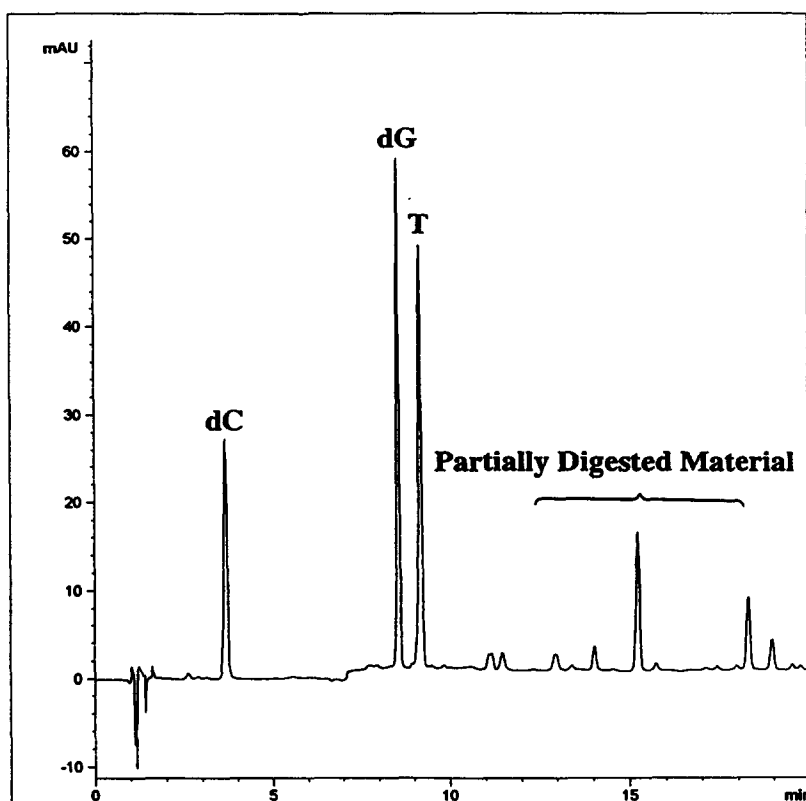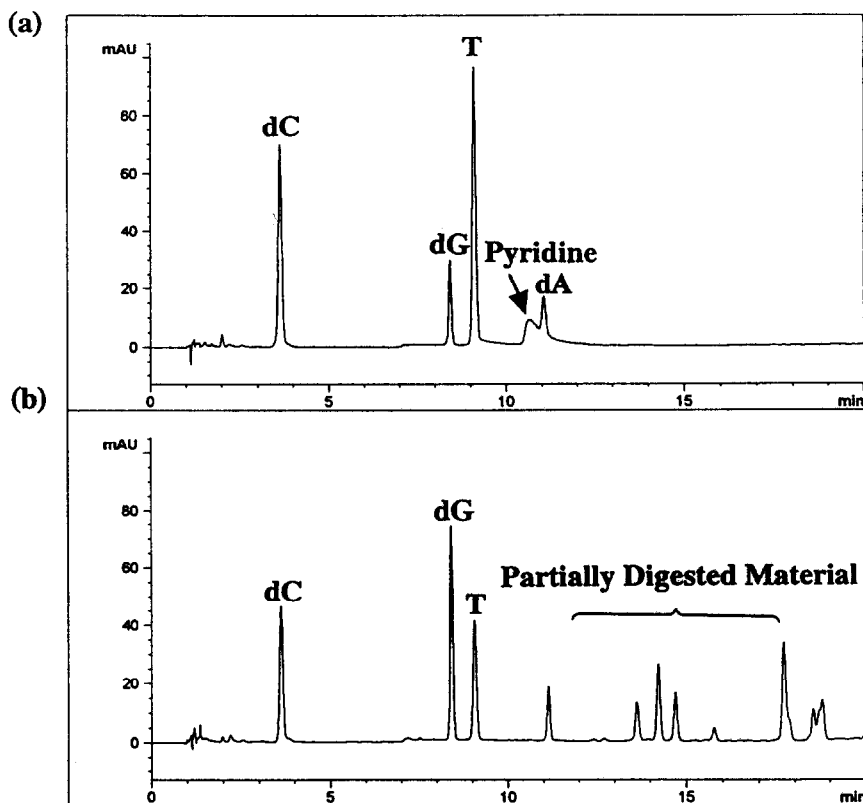

Figure 1
Chromatogram of a directly digested 21-base phosphorothioate sequence (0.67 mg ml^{-1}). The sample was incubated over a 96-h period with aliquots of enzymes introduced at 48 and 72 h following the first addition.

accuracy of the calculated base composition for ISIS 2922:0245, shown in Table 3.

THF/water/methylimidazole/iodine treatment

The treatment employed by Porritt and Reese [13] for the desulphurization and subsequent characterization of phosphorodithioate oligodeoxyribonucleotides was adapted for the analysis of longer phosphorothioate sequences. It was noted during the development stage that increasing the relative water ratio from three volume equivalents to four promoted more effective desulphurization. Validation of this modified method in Table 4 confirms it to be adequate for routine analysis, as indicated by the 1.1–1.3% and 0.6–2.0% RSD values between days and lots, respectively. Accuracy of the base ratios calculated for multiple ISIS 2922 lots was confirmed by comparison with the ratio calculated for ISIS 2922:0273, a lot which was independently sequenced via matrix-assisted laser desorption

Figure 2
Chromatograms depicting (a) incomplete removal of pyridine from a 20-base phosphorothioate oligonucleotide sample (0.5 mg ml^{-1}) containing deoxyadenosine and (b) inefficient oxidation of a 21-base phosphorothioate oligonucleotide sample (0.55 mg ml^{-1}) resulting in a range of incompletely digested material. Samples were oxidized using the pyridine/iodine treatment.

Table 2
Base composition of ISIS 2922 and Afovirsen (ISIS 2105) desulphurized with iodine/pyridine

Sample	Concentration* (mg ml^{-1})	Incubation time (h)	Base ratio ($n = 3$) dC	dG	T	dA
ISIS 2922:0273						
Day 1	0.55	15	6.1 ± 0.1	3.7 ± 0.4	11.1 ± 0.3	0.0
Day 2	0.55	15	6.0	4.6 ± 0.1	10.4	0.0
ISIS 2922:089†	0.56	8.33	6.1	5.2	9.7	0.0
	0.56	12	5.9	5.4 ± 0.2	9.6 ± 0.1	0.0
ISIS 2105:018‡	0.50	15	7.9 ± 0.01	1.8 ± 0.1	9.2 ± 0.1	1.1

* Concentrations expressed do not account for water content of ISIS 2922 or ISIS 2105.
† For the 12 h ISIS 2922:089 sample, ($n = 2$). Expected ratio for ISIS 2922 is 6 dC:5 dG:10 T:0 dA.
‡ The expected base ratio for ISIS 2105 is 8 dC:2 dG:9 T:1 dA.

Table 3
Effect of iodine–sulphur concentration on the efficiency of phosphorothioate oxidation

Sample	Equivalent* of iodine–sulphur	Incubation time (h)	Base ratio dC	dG	T	dA
ISIS 2105†	62:1	16.5	6.4	1.8	10.9	1.0
ISIS 2922:089‡	62:1	16.5	4.9	4.6	11.5	0.0
ISIS 2922:0245	335:1	12	5.6	5.1	10.3	0.0
	335:1	26	5.9	5.2	9.9	0.0

* Concentrations of iodine are expressed in terms of equivalents of oligonucleotide sulphur and do not account for water content of ISIS 2922 or ISIS 2105.
† Metaperiodate not removed prior to analysis. The expected base ratio for ISIS 2105 is 8 dC:2 dG:9 T:1 dA.
‡ Expected ratio of ISIS 2922 is 6 dC:5 dG:10 T:0 dA.

Table 4
Accuracy and day-to-day/lot-to-lot precision for oxidation by tetrahydrofuran/water/methylimidazole/iodine treatment

Sample	Incubation (h)	Base ratio (n = 3)			
		dC	dG	T	dA
ISIS 2922:0273*					
Day 1	15.0	6.1	4.8 ± 0.1	10.1 ± 0.1	0.0
Day 2	15.0	6.2 ± 0.1	4.8 ± 0.1	10.0 ± 0.1	0.0
ISIS 2922:0276†	15.0	6.1	4.6	10.2	0.0
ISIS 2922:0277	15.0	6.1 ± 0.1	4.7 ± 0.1	10.2	0.0
ISIS 2922:0278	15.0	6.1 ± 0.1	47. ± 0.1	10.2	0.0

*Expected ratio for ISIS 2922 is 6 dC:5 dG:10 T:0 dA as confirmed by matrix-assisted laser desorption ionization mass spectrometry of ISIS 2922:0273 enzymolysis products.
†The sequence of ISIS 2922:0276 has been confirmed by matrix assisted laser desorption ionization mass spectrometry.
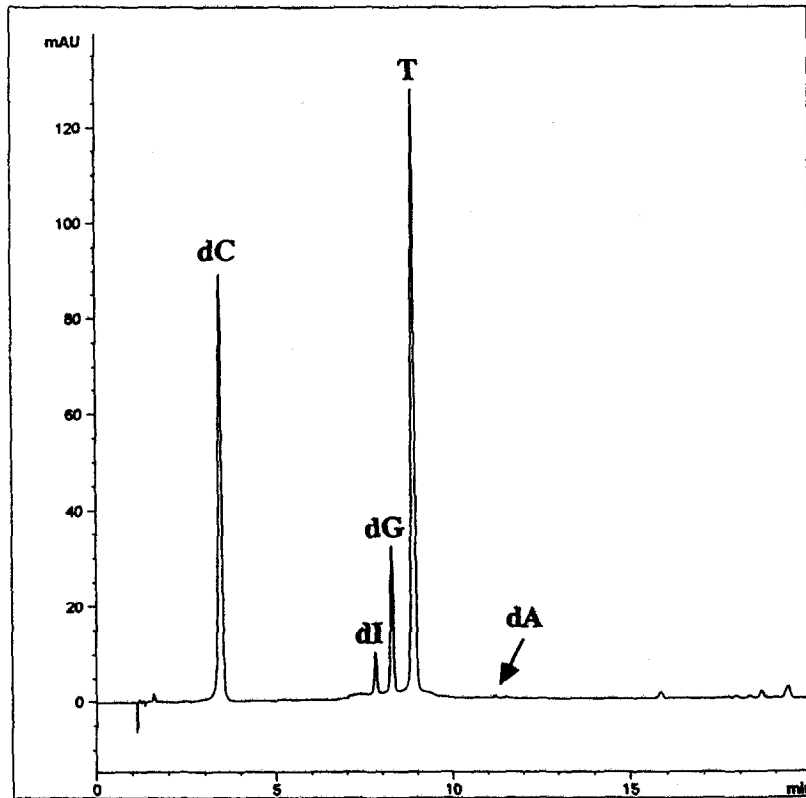
Figure 3
Chromatogram of digestion products of a 20-base phosphorothioate oxidized using the THF/H$_2$O/iodine treatment. Note the presence of the deamination product of 2'-deoxyadenosine, 2'-deoxyinosine.

ionization time-of-flight mass spectrometric (MALDI-TOF/MS) analysis of the products from an enzymolysis reaction [16].

Deaminase that is occasionally present in snake venom phosphodiesterase may convert the 2'-deoxyadenosine to 2'-deoxyinosine, represented by a peak with retention time between that of dC and dG (see Fig. 3). The actual concentration for deoxyadenosine is derived from the contributions of both deoxyinosine and any remaining deoxyadenosine. Table 5 demonstrates that the base com-

Table 5
Robustness of the THF/H$_2$O/iodine treatment for base composition analysis of samples containing 2'-deoxyadenosine

Sample	Base ratio (n = 3)			
	dC	dG	T	dA + dI
ISIS 2105*	7.8 ± 0.1	2.0 ± 0.1	9.2 ± 0.1	1.0
ISIS 2302†	8.0 ± 0.1	4.9 ± 0.1	3.1 ± 0.2	4.0

*Expected ratio for ISIS 2105 is 8 dC:2 dG:9 T:1 dA.
†Expected ratio for ISIS 2302 is 8 dC:5 dG:3 T:4 dA.

positions calculated in this way are, in fact, accurate for the 20-base sequences analysed. Precision of the quantitation following this treatment does appear to be sensitive to the actual amount of the individual nucleoside present. For example, the value for thymidine in ISIS 2302 appears to be less precise than the estimate for thymidine in ISIS 2105 (afovirsen sodium). It is also possible that this discrepancy reflects a particular structural conformation of thymidine in ISIS 2302 vs in afovirsen since in the case of deoxyadenosine quantitation apparently does *not* depend on the relative amount of the nucleoside present for either compound.

A series of single-deletion sequences of the 21-base oligomer, ISIS 2922, were analysed to address the question of whether accessibility to or reaction with oxidation reagents varied with base type or location. Potential use of base composition analysis to detect manufacturing process-related sequence failures was also evaluated. Table 6 lists the actual expected base ratio for the individual samples analysed according to the synthesis. The ratios were calculated in two different ways. Assuming no base deletion, or 21 total bases as is expected for samples of ISIS 2922, the sensitivity of the method to detect a real base discrepancy can be evaluated. Quantitation of the nucleosides in samples CC and EE indicates an over-estimation of thymidine and underestimation of deoxyguanosine according to the usual, expected ratio of ISIS 2922 (6 C:5 G:10 T). Assuming a total of 20 bases present (as would result from a base deletion) and without prior knowledge of the actual base composition, a ratio of 6 C:4 G:10 T is calculated. This was confirmed to be correct by reference to the synthesis cycle. A deoxycytidine deletion was also correctly detected for sample LL. How-

ever, for sample KK, a thymidine deletion was not detectable by simply assuming the presence of all 21 bases, as would be expected for a full ISIS 2922 sequence. Assuming the presence of a base deletion (or 20 total bases) did confirm the absence of thymidine. It is important to note that generally information regarding the total expected nucleosides in a sequence (through gel electrophoresis or mass spectrometry) would be known prior to calculation of the base composition. With this information available and in the presence of a detectable concentration of failure sequences, it is possible to reliably detect some base deletions due to synthesis error.

Conclusions

While conditions have been achieved for the direct digestion of ISIS 2922, this approach is quite time-consuming and, therefore, not suitable for routine base composition analysis. In general, accuracy of the calculated base composition relies on the efficiency of the oxidation step and, subsequently, the extent of enzymatic degradation of the oligonucleotide. In addition, methods that employ pyridine require stringent purification steps to avoid potential interference with the quantitation of deoxyadenosine.

THF and methylimidazole appear to improve reactivity of the iodine by converting the sulphur into a better leaving group. This is supported by the fact that fewer equivalents of iondine are required to oxidize the phosphorothioate in the presence of THF and methylimidazole. Desulphurization via $THF/H_2O/$ methylimidazole and iodine has been *validated* in the present work, according to the guidelines of the United States Pharmacopeia [17], as a particularly rugged and reliable approach

Table 6
Base composition analysis results for single base deletion sequences of ISIS 2922

Sample ($n = 3$)	Calculated base ratio (no. bases = 21)	Calculated base ratio (no. bases = 20)	Expected base ratio*
2922:CC			
Day 1	6.3 ± 0.1 C:3.9 ± 0.1 G:0.8 ± 0.2 T	6.0 ± 0.1 C:3.7 ± 0.1 G:10.3 ± 0.2 T	6 C:4 G:10 T
Day 2†	6.0 C:3.8 G:10.2 ± 0.1 T		
2922:EE†	6.4 C:3.8 ± 0.2 G:10.7 ± 0.1 T	6.1 C:3.7 ± 0.2 G:10.2 ± 0.1 T	6 C:4 G:10 T
2922:KK	6.3 ± 0.1 C:4.8 ± 0.1 G:9.9 ± 0.2 T	6.0 ± 0.1 C:4.6 ± 0.1 G:9.4 ± 0.2 T	6 C:5 G:9 T
2922:LL	5.2 C:4.9 ± 0.1 G:10.9 ± 0.1 T	5.0 ± 0.1 C:4.7 ± 0.1 G:10.4 ± 0.1 T	5 C:5 G:10 T
ISSIS 2922	6.1 C:4.8 ± 0.1 G:10.1 ± 0.1 T	5.8 C:4.6 ± 0.1 G:9.6 ± 0.1 T	6 C:5 G:10 T

* Expected ratio is derived from the actual synthetic sequence.
† ($n = 2$).

for mediating *routine* base composition analysis of *phosphorothioate* oligonucleotides. The method is accurate, has high day-to-day and lot-to-lot precision, provides a means for rapid determination of base composition with minimal sample consumption, and meets FDA requirements for establishment of phosphorothioate oligonucleotide therapeutic agents. Finally, the determination of nucleoside concentrations by HPLC is a sensitive technique capable of discerning single base composition differences for the detection of potential synthesis process failures. Since this same approach has been used in our laboratory to facilitate sequencing of such molecules, there exists potentially wider application for the oxidation approach in terms of routine analyses of phosphorothioate oligonucleotides throughout the biopharmaceutical industry.

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