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Quantitative analysis of phosphorothioate oligonucleotides in biological fluids using fast anion-exchange chromatography

A. J. Bourque and A. S. Cohen*

Hybridon, Inc., One Innovation Drive, Worcester, MA 01605 (USA)

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ABSTRACT

Phosphorothioate oligonucleotides are potentially useful as anti-viral drugs. Classical DNA extraction methods are not as effective on short single-stranded DNA as with longer double-stranced chains. The classical method of phenol–chloroform extraction followed by ethanol precipitation is difficult to quantify, thus monitoring of the pharmacological disposition of these compounds is subject to error. A method has been devised and validated for extraction and analysis of modified oligonucleotides from biological fluids such as urine and serum based on protein kinase digestion and phenol–chloroform extraction. Due to the high native ultraviolet absorbance of the oligomers, detection limits in the low ppb range were obtained without derivatization.

INTRODUCTION

Oligodeoxynucleotides (ODNs) which are complementary to a specific RNA sequence ("antisense" recognition) have been shown to effectively inhibit the expression of the targeted gene [1,2]. One of the challenges of antisense therapy is to obtain high intracellular concentrations of the antisense ODN. The hydrophilic nature of the single-stranded ODN slows cellular uptake, and once in the cell, the oligomers are susceptible to exonuclease activity. One approach to circumvent these phenomena is to replace a non-bridging oxygen in the internucleotide phosphate linkage with a sulfur atom. These analogs of DNA, known as phosphorothioates (SODNs), are more lipophilic and exhibit longer lifetimes *in vivo* due to their nuclease resistance. More importantly, Watson-Crick hybridization, which is the basis for complementary interaction, is not affected by this substitution.

The baseline resolution of single-stranded DNA (ssODN) of 10–30 bases in length which differ by a single base is easily accomplished by reversed-phase [3] and anion-exchange high-performance liquid chromatography (HPLC) [4]. However, phosphorothioate DNA is much more difficult to resolve. Reversed-phase HPLC can be used to separate only very small SODNs (< 10, bases) and anion-exchange supports begin to fail above 20 bases [5,6].

The SODNs have been successfully extracted from serum [7] and tissue homogenates [8]. Following the digestion of proteins using protein kinase, the DNA was isolated using phenol–chloroform–isoamyl alcohol extraction followed by ethanol precipitation [9]. No recovery studies were reported at trace levels, and the resulting chromatograms [7] were overwhelmed by a large phenol peak. A bioassay has been reported in which the sample containing the antisense phos-

* Corresponding author.

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phodiester DNA is directly injected into the HPLC system with minimal sample handling. The antisense DNA and its degradation products are extracted on-line from the sample matrix using Pinkerton-type restricted access media, and the retained components are then backflushed to the analytical column [10].

This paper describes the development of an analytical method for the monitoring of phosphorothioate oligonucleotides in biological media. The method contains a protein digestion step, a phenol–chloroform extraction, two *sec.*-butanol extractions and one diethyl ether extraction. The concentrated extract is then diluted with buffered formamide and analyzed by fast anion-exchange chromatography. Detection is via UV absorption at 270 nm and quantification is performed using an internal standard. Analytical figures of merit such as detection limits, linear range of detection, recovery, precision and accuracy are addressed. Single-blind spiked studies are performed to validate the method.

EXPERIMENTAL

Chemicals and reagents

The 10- and 25-mer SODNs were synthesized in-house. Water and acetonitrile were HPLC grade (J. T. Baker, Phillipsburg, NJ, USA). Human serum (male) was obtained from Sigma (St. Louis, MO, USA). Lithium bromide was obtained from Fluka (Ronkonkoma, NY, USA). Spectrophotometric-grade formamide was obtained from Aldrich (Milwaukee, WI, USA). Proteinase K was obtained from Applied Biosystems (Foster City, CA, USA). Chloroform, phenol, isoamyl alcohol and other chemicals were obtained from J. T. Baker.

Instrumentation and equipment

Semi-preparative HPLC was performed on a Waters system consisting of two Model 510 pumps, a Model 717 WISP autosampler, an automated gradient controller and a Model 490E programmable-wavelength detector (Waters Chromatography, Division of Millipore, Milford, MA, USA). The SODNs were purified via anion-exchange HPLC on a 250 mm × 9 mm I.D. Nucleopak PA-100 (Dionex, Sunnyvale, CA, USA). Fractions were collected on a Model 202 fraction collector (Gilson Medical Instruments, Middleton, WI, USA). Samples were homogenized on a Model G-560 vortex-mixer (Scientific Instruments, Bohemia, NY, USA). Serum digestions were performed in a Model GP-200 constant-temperature bath (Neslab Instruments, Newington, NH, USA). Samples were centrifuged in a Model 5402 refrigerated centrifuge (Eppendorf, Brinkman Instruments, Westbury, NY, USA). Analytical anion-exchange HPLC was performed on a Series II 1090 liquid chromatograph (Hewlett-Packard, Germany). The data were acquired and stored on an AcerPower 486/33 computer (Acer American, San Jose, CA, USA) through a Model 970 analog-to-digital converter (PE Nelson, Cupertino, CA, USA).

Purification of phosphorothioate oligonucleotides

The crude SODNs containing a DMT label were deprotected on an Oligo-Pak oligonucleotide purification column (MilliGen/Biosearch, Division of Millipore) following the manufacturer's protocol. The sample was then purified via semi-preparative anion-exchange HPLC. The main peak was collected and desalted on the Oligo-Pak cartridge. The solution containing the desalted SODN was evaporated to dryness and stored at 4°C. The purity of the SODN was checked via anion-exchange HPLC and capillary electrophoresis and was determined to be greater than 98% pure by area. Concentrations were determined by UV spectroscopy using calculated molar absorptivity factors.

Optimization of digestion time

Aliquots (250 μl) of human serum were spiked with 250 ng of each oligomer. A 250-μl volume of DNA extraction buffer (50 m*M* sodium dodecylsulfate, 10 m*M* NaCl, 20 m*M* Tris, 10 m*M* EDTA, pH 8.0) and 20 μl of 10 mg/ml proteinase K were added to the spiked serum. The final volume of the sample was brought to 600 μl with sterile water. Three samples per time point were heated at 60°C for 60, 120 and 180 min. To each sample

was added a 600-μl aliquot of a phenol–chloroform–isoamyl alcohol solution. The sample was then vortex-mixed for 15 s and centrifuged for 2 min at 15 800 *g*. The supernatant was collected, and 1000 μl of *sec*-butanol [2 m*M* dithiothreitol (DTT)] were added. The sample was again vortex-mixed for 15 s and centrifuged for 1 min at 15 800 *g*. The supernatant (*sec*-butanol) was aspirated and a second 1000-μl aliquot of *sec*-butanol (2 m*M* DTT) was added to the sample, which was then vortex-mixed, centrifuged and aspirated as above. A 500-μl aliquot of diethyl ether was added, vortex-mixed, centrifuged and aspirated as above. The sample at this point has been reduced to a 30–50 μl volume. A 200-μl volume of 25 m*M* Tris, 2 m*M* EDTA, 2 m*M* DTT in 30% formamide was added, vortex-mixed to wash the walls of the Eppendorf tube and spun for 20 s at 14 000 rpm. The diluted extract was transferred to HPLC autosampler vials and duplicate injections of 100 μl were performed. The percentage recovery was calculated by comparison of sample peak areas to an external standard.

Single-blind spiked serum

A calibration curve which bracketed the region of interest was generated from known concentrations of the 25-mer spiked into serum containing 125 ng of the 10-mer (internal standard). Five 250-μl aliquots of serum were spiked with 125 ng of 10-mer and an unknown quantity of the 25-mer. All other excipients were added as above to a final volume of 605 μl. All samples and standards were digested, extracted and concentrated at the same time and the analysis was performed with the standards bracketing the samples. The spiked amounts were determined from the internal standard calibration curve.

RESULTS AND DISCUSSION

The resolution of phosphorothioate oligomers of 25 bases and less was accomplished in less than 4 min by fast anion-exchange chromatography. This allowed for the rapid monitoring of SODNs in biological fluids. The high native UV absorbance of these SODNs allowed for detection of concentrations as low as 20 ppb (200-μl injection, 4 ng on-column). The selectivity of the *sec*-butanol extractions and the high affinity of phosphorothioates for the anion-exchange support yielded chromatograms which were virtually identical to injection of the standards.

Resolution of phosphorothioates

Mobile phase. The elution of phosphorothioates from strong anion-exchange supports required much higher concentrations of salt than expected based on elution data for phosphodiester ODNs. The anions which are used for elution of phosphodiester DNA, *i.e.* phosphate, sulfate and perchlorate, produced excessively long run times and broad peak shapes. We have found that most oxygen-based anions are poor displacers of SODNs from strong anion-exchange supports. Halides, particularly bromide and chloride, are much more efficient displacers [11]. A 1 *M* LiBr gradient was approximately the same strength as a 2 *M* LiCl gradient with respect to elution volumes. Although chloride gave higher resolution of closely eluting peaks, bromide yielded narrower peak widths and thus higher mass sensitivity. Addition of formamide decreased the capacity factor without greatly affecting the resolution of closely eluting peaks. A mobile phase of 30% formamide containing 1 *M* LiBr yielded narrow bands and a 3.5-min analysis at 1 ml/min. The formamide also helped to solubilize components in the serum extract which otherwise might have been irreversibly adsorbed to the stationary phase.

Stationary phase. Only hydrophilic, polymer-based, non-porous, strong anion-exchange supports were investigated. It was found that the resolution of SODNs (3–25 bases in length) was virtually independent of the length of the column and the steepness of the gradient. The oligomers did not appear to partition, but were displaced at distinct concentrations of anion. Thus, it was assumed that a short anion-exchange column with a rapid bromide gradient would give the fastest sample turnover. Sensitivity was gained due to concentration of large sample aliquots on the head of the column and rapid elution in narrow

peak volumes. The flow-rate of 1 ml/min was optimized to yield the shortest analysis time *and* the highest sensitivity. The linear range of detection under optimized separation conditions was determined from 50 ppm to the detection limit. Both oligomers exhibited linear behavior in this concentration range with correlation coefficients of ≥ 0.99998 (Fig. 1).

Temperature effects. Increased column temperature, which increases efficiency and reduces retention on reversed-phase supports, had nearly the opposite effect here. The DNA elution volumes increased slightly with increased temperatures [12]. To minimize the possibility of secondary structure, all chromatograms were run at 70°C.

Sample preparation

Digestion protocol. The digestion time was studied between 60 and 180 min. The average recovery of the two SODNs was 84 ± 2% at 60 min, 90 ± 1% at 120 min, and 79 ± 3% at 180 min. A digestion time of 90 min was chosen as convenient. The digestion time could be further reduced by increased concentrations of protein kinase [7].

Extraction of SODNs from biological matrices. This method was generated to overcome the non-quantifiable nature of ethanol precipitation. We had recognized the potential of utilizing acid precipitation for the isolation of SODNs in the presence of native DNA. SODNs readily precipitate at pH 1 while normal phosphodiester DNA of the same molecular mass does not, presumably due to differences in the pK_a of the two groups [13]. However, this was not practical because of the equilibrium solubility of SODNs at concentrations below 1 ppm. SDS and/or urea from the digestion buffer increased the solubility of SODNs while salt (NaCl) suppressed the equilibrium. In addition, the pH precipitation was chain length-dependent. This created problems when attempting to use an internal standard since the precipitation behaviors were quite different. We decided that any precipitation step in the method would introduce unacceptable variance to the

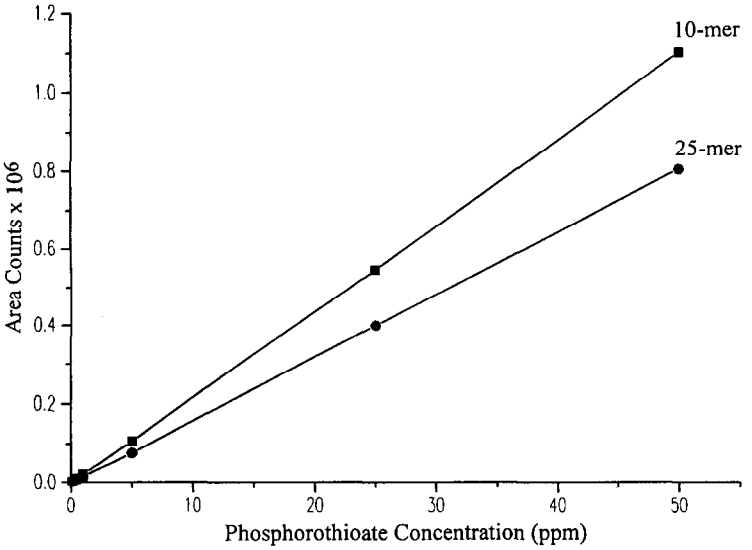

Fig. 1. Calibration curve to establish linear range of detection. The area of the 10- and 25-mer is plotted *versus* their concentrations in ppm. The calibration curves are as follows: 10-mer, $y = 22.1074x - 2146$, $r^2 = 0.99998$; 25-mer, $y = 16.1588x - 2029$, $r^2 = 0.99998$. Conditions: 50 mm × 4 mm I.D. Nucleopak PA-100, 25 μl injection volume. Mobile phase: (A) 30% formamide, 25 m*M* Tris buffer (pH 8.5); (B) 30% formamide, 25 m*M* Tris buffer (pH 8.5) containing 1 *M* LiBr. Gradient: 20% B to 100% B over 2.5 min, 1 ml/min at 70°C.

analysis. *sec*-Butanol extractions provide a convenient means to concentrate dilute aqueous samples of DNA. Two 1-ml *sec*-butanol extractions removed all traces of phenol, lipid-soluble components of the digested serum and most of the water. A 500-μl diethyl ether extraction was performed to remove the small amount of *sec*-butanol in the concentrated extract. The resulting solution (*ca.* 30–50 μl) was concentrated in native DNA, SODNs and salt. A 30% formamide solution buffered at pH 8.5 containing DTT was added to prevent salting out of the SODNs and to facilitate recovery of the SODNs from the Eppendorf tube.

The extraction protocol worked well with urine which contained 1 ppm levels of both oligomers. Since urine contains very low amounts of protein, no digestion protocol was used. The recovery of the three oligonucleotides was 91 ± 3% and the chromatogram was virtually identical to injection of the standards with the exception of the solvent front (Fig. 2). This is due to the selectivity of the extraction protocol and the high affinity of the SODNs for the anion-exchange support.

One of the more interesting phenomena observed during these studies was the partial oxidation of the SODNs during sample work-up. Replacement of a single sulfur atom with an oxygen atom resulted in a dramatic shift of retention. If extensive oxidation had occurred, a Gausian profile of oligomers was seen (Fig. 3). This profile could be generated through the use of hydrogen peroxide or 2,3-epoxypropanol (glycidol) and has been more extensively studied by others [10]. We believed this problem was due to trace peroxides present in the *sec*-butanol and diethyl ether. The addition of 2 m*M* DTT to the *sec*-butanol, diethyl ether and formamide buffer minimized this phenomena.

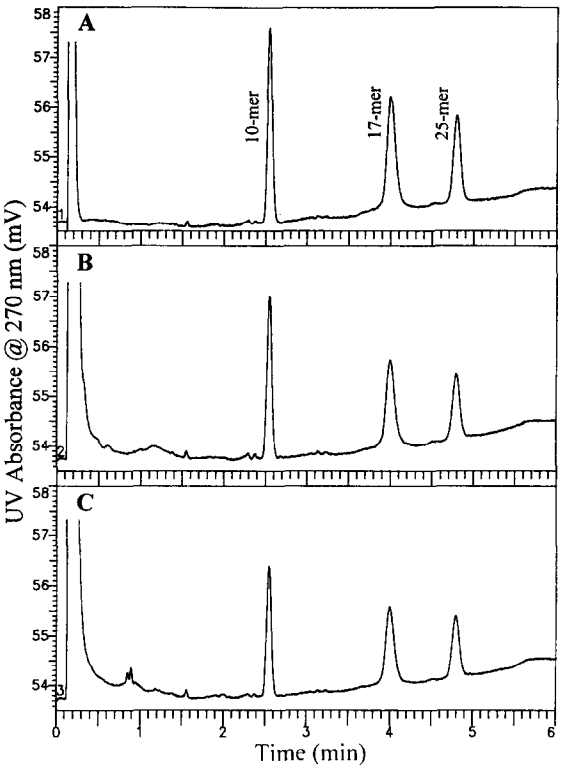

Fig. 2. Anion-exchange HPLC of three SODNs extracted from urine and serum. Each sample (250 μl) was spiked with 250 ng of 10-, 17-, and 25-mer SODN. (A) Urine extract (no digestion); (B) serum extract following digestion; (C) standards in 30% formamide buffer. Conditions: 50 mm × 4 mm I.D. Nucleopak PA-100, 50 μl injection volume. Mobile phase: (A) 20% acetonitrile, 25 m*M* Tris buffer (pH 8.5); (B) 20% acetonitrile, 25 m*M* Tris buffer (pH 8.5) containing 2 *M* LiCl. Gradient: 10% B to 100% B over 5 min, 2 ml/min at 70°C.

Analysis

Quantification of SODNs. A 10-mer SODN was chosen as an internal standard because exonuclease degradation of the 25-mer would only produce a 10-mer after extensive degradation. The base sequence of the 10-mer was chosen as a random mixture of cytosine and thymidine, since these bases chromatograph with higher efficiency than the purines and are not prone to depurination and other phenomena which would limit their utility as internal standards. Addition of the internal standard increased the precision of quantification by accounting for the recovery of SODN from the matrix and accounting for the variation in the final extracted volumes. The calibration curve generated from serum extracts bracketed the region of interest and exhibited excellent linearity (the calibration curve was $y = 4.5093 \cdot 10^{-3} x - 0.0007$, $r^2 = 0.9994$). The estimated limit of detection in terms of concentra-

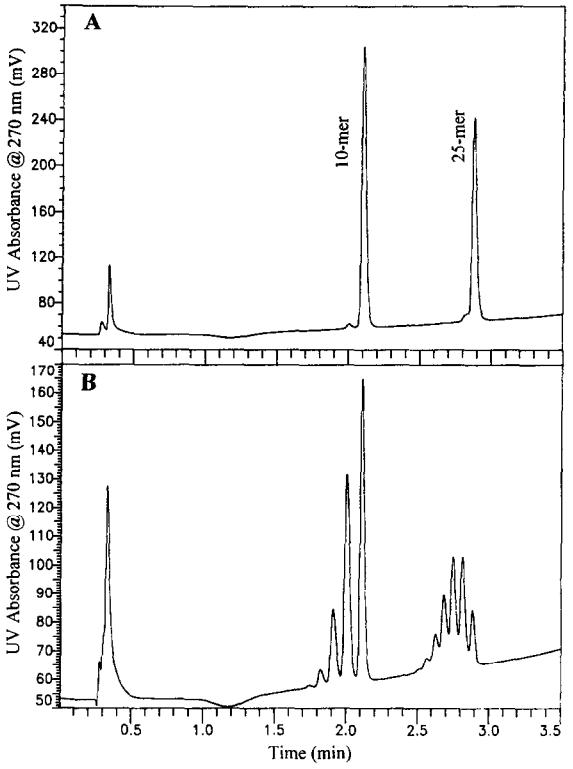

Fig. 3. Fast anion-exchange HPLC of phosphorothioate oligomers and oxygenated defects. (A) 25 ppm sample 10-mer and 25-mer as the pure phosphorothioate standards. (B) The same sample after 10-min reaction with glycidol at 100°C. Conditions identical to those of Fig. 1.

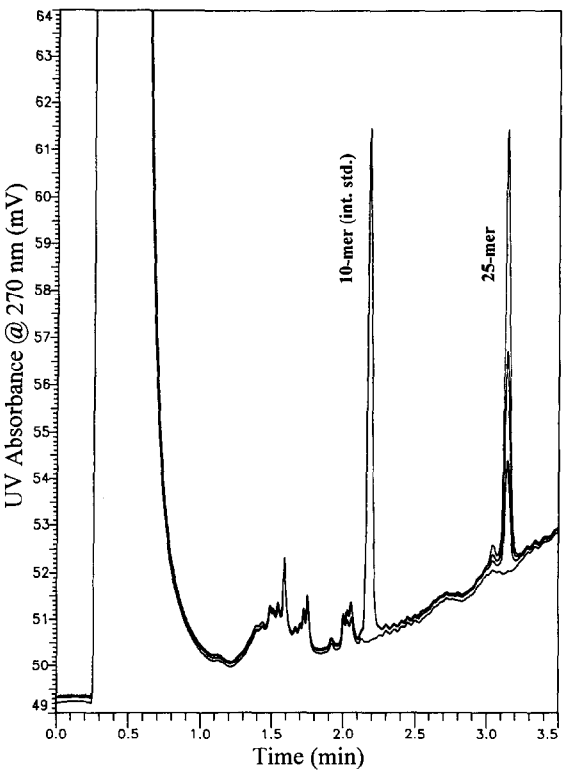

Fig. 4. Overlay of extracted standards from human serum at trace levels. The overlaid samples represent the blank, 200-, 400-, and 800-ppb spikes of 25-mer in each sample. The 10-mer is present at 500 ppb in each sample. Conditions are identical to those of Fig. 1, except injection volume was 100 μl.

tion was one fifth of the 200-ppb spike which had been extracted from serum (Fig. 4). This corresponds to 4 ng of 25-mer (200-μl injection volume) at a signal-to-noise of 3.

A single-blind spiking experiment was used to validate the method for the determination of SODNs in human serum. The samples and standards were digested, extracted, concentrated, and analyzed. The samples were bracketed by standards. The mass of 25-mer spiked into the serum was determined using the internal standard calibration curve (Table I). Carry-over of SODNs from sample to sample was minimized by the use of 30% formamide in the mobile phase and extending the LiBr gradient beyond that needed for elution of all SODN species. The absence of matrix or injection-based artifacts was reflected in the percentage relative error of the determined mass present in each serum sample (Table I).

CONCLUSION

The use of antisense pharmaceuticals for the regulation of viral expression is in its infancy.

TABLE I

SINGLE BLIND SPIKED SAMPLE DATA

Sample identification	Ratio[a]	Determined[b] (ng)	Actual (ng)	R.E.[c] (%)
Spike 1	0.3545	79	80	−1.3
Spike 2	1.1635	258	250	+3.2
Spike 3	0.5465	121	125	−3.2
Spike 4	1.4129	195	190	+2.6
Spike 5	0.2129	47	50	−5.3

[a] Area ratio of 25-mer divided by 10-mer, average of two injections.

[b] From plot of ng 25-mer *versus* internal standard area ratio.

[c] Percent relative error of determined value from actual mass.

Fast, reliable analytical methods are needed for the accurate monitoring of blood levels of these compounds. The proposed method allows the selective monitoring of SODNs in serum and urine at trace levels.

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