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Quantitative analysis of phosphorothioate oligonucleotides in biological fluids using direct injection fast anion-exchange chromatography and capillary gel electrophoresis

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Abstract

The analysis of antisense phosphorothioate DNA (SODN) in human plasma via direct injection using anion-exchange high-performance liquid chromatography (AE-HPLC) is presented. The method relies on the ability to selectively extract phosphorothioate DNA from undigested serum, plasma and urine on anion-exchange resins. The automated HPLC method can analyze a sample every 5 min with a limit of detection of 50 ng/ml (ppb). The DNA was collected, desalted and analyzed by capillary gel electrophoresis. Due to the high resolving power of this technique, a qualitative assessment of enzymatic degradation of the antisense oligonucleotide can be made.

1. Introduction

Antisense DNA technology offers the means to modulate cell replication based on the hybridization of synthetic DNA with messenger RNA from an essential gene. In this manner, oncogene expression and virus replication can be suppressed. The elegance and simplicity of this approach implies that once a gene responsible for an undesirable symptom is located, synthetic DNA can be produced to reduce the symptom. This synthetic DNA must be approximately 15–35 bases in length for specificity to the targeted gene. The antisense DNA must be modified to prevent enzymatic digestion *in situ*. One of the most conservative modifications is to replace one of the non-bridging oxygen atoms in the phosphate backbone with a sulfur atom. These DNA analogs are known as phosphorothioates.

Very few methods have been published for the quantification of low levels of single-stranded synthetic DNA in biological fluids such as plasma, urine, cellular extracts and tissue homogenates [1]. Most require a significant amount of sample handling including time-consuming protein digestion, phenol/chloroform extraction and ethanol precipitation of the extracted DNA [2]. We have modified this approach to accurately quantify phosphorothioate DNA in serum and urine, but it still remains a relatively laborious procedure [3].

We were interested in increasing the sample throughput and reducing the amount of sample handling required for a bioassay. An on-line HPLC sample clean-up was used to study the degradation kinetics of unmodified antisense oligonucleotides in cell culture media [4]. This

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method used an HPLC equipped with a switching valve which contained an internal surface reversed-phase pre-column. The oligonucleotides were extracted from the media and then back-flushed to the analytical column.

We attempted to use this technique to analyze human plasma spiked with various concentrations of phosphorothioate DNA, but consistently ran into problems of back-pressure due to protein precipitation when the oligonucleotides were eluted to the anion-exchange column using acetonitrile as the organic modifier.

In the present paper a completely biocompatible on-line HPLC sample clean-up was devised in which no organic solvents were used. The linearity, limit of detection, accuracy, and precision were determined. The HPLC effluent was monitored using a fraction collector and after desalination, the sample extract was analyzed by capillary gel electrophoresis.

2. Experimental

2.1. Chemicals and reagents

The 25-mer SODN was synthesized in-house. Water and acetonitrile (ACN) were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Human serum (male) was obtained from Sigma Chemical (St. Louis, MO, USA). Uniflo 13-mm diameter 0.2-μm cellulose triacetate disposable syringe filters were obtained from Schleicher and Schuell (Keene, NH, USA). Solid-phase extractions were performed on Supelclean SAX SPE columns (Supelco, Bellefonte, PA, USA). Lithium bromide and other chemicals were obtained from Fluka (Ronkonkoma, NY, USA).

2.2. Instrumentation and equipment

Analytical anion-exchange chromatography (AE-HPLC) was performed on a Series II 1090m liquid chromatograph (Hewlett-Packard, Germany) equipped with a switching valve and heated column compartment. Anion-exchange separations were performed with a stainless steel 20 × 2 mm I.D. guard column (UpChurch Scientific, Oak Harbor, WA, USA) hand-packed with spherical 13-μm Dionex Nucleopak PA-100 support. Titanium frits (2-μm pores) were placed on either end of the column. Fractions were collected on a Model 202 fraction collector (Gilson Medical Instruments, Middleton, WI, USA). 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).

The capillary electrophoresis apparatus with UV detection and the preparation of the gel-filled capillary for the separation of DNA molecules have been described previously [5,6]. A 30-kV, 500-μA direct current high voltage power supply (Model ER/DM; Glassman, Whitehouse Station, NJ, USA) was used to generate the potential across the capillary. UV detection of phosphorothioates at 267 nm was accomplished with a Spectra 100 (Spectra-Physics, San Jose, CA, USA). Samples were dialyzed *vs.* distilled water on 0.025-μm cellulose triacetate membranes (Millipore, Bedford, MA, USA).

2.3. Gel-filled capillaries

Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) with inner diameter of 75 μm, outer diameter of 375 μm, effective length of 9 cm and total length of 19 cm was treated with (methylacryloxypropyl)trimethoxysilane (Petrarch Systems, Bristol, PA, USA) and then filled with a degassed solution of 18% polymerizing linear acrylamide in 30% (v/v) formamide media (0.1 *M* Tris-borate, 2.5 m*M* EDTA-2Na$^+$ buffer (pH 8.3) containing 7 *M* urea). Polymerization was achieved using ammonium persulfate/TEMED chemistry. An electric field of 600 V/cm was applied resulting in a current of 2–3 μA.

2.4. Preparation of mobile phase

The AE-HPLC method requires binary solvent gradient elution at 50°C. Mobile phase A consisted of 25 m*M* Tris, 1 m*M* EDTA adjusted to pH 7.0. Mobile phase B was 25 m*M* Tris, 1

m*M* EDTA (pH 7) containing 2 *M* LiBr. The initial mobile phase was held at 250 m*M* LiBr (pH 7) at 0.7 ml/min for 0.5 min and then brought to 2000 m*M* LiBr over 2 min and held for 1.0 min. The flow-rate was then doubled and the composition was returned to initial conditions. After 1 min the flow was returned to 0.7 ml/min and the next sample was injected. Using this mobile phase program, a sample can be analyzed every 5 min.

2.5. Preparation of analytical standards

The 25-mer phosphorothioate oligonucleotide which was used to validate this method was synthesized in-house and purified by preparative AE-HPLC. The sample was then desalted on a C_{18} column with a triethylammonium acetate–acetonitrile mobile phase. The desalted oligonucleotide was lyophilized and reconstituted in 1 ml of 20 m*M* Tris, 2 m*M* EDTA (pH 8.5). A 10-μl aliquot of this sample was diluted to 1 ml and the absorbance at 267 nm was determined. The absorbance and dilution factor were multiplied by 40.8 μg/a.u. to obtain the concentration of the analytical standard. Serial dilutions in buffer, urine or serum were performed to obtain the calibration curves and spiked samples.

2.6. Double blind spiked serum

A calibration curve which bracketed the region of interest was generated by serial dilution of known concentrations of the 25-mer spiked into serum. Four 1-ml aliquots of serum were spiked with an unknown quantity of the 25-mer. The sample was split in half and stored at 4°C. The other half was sent to a collaborating laboratory which had received full documentation of the analytical protocol. After 10 days of storage at 4°C, the sample was filtered and analyzed with standards bracketing the samples. The spiked amounts were interpolated from the standard calibration curve. The data obtained by the two separate laboratories were compared (Table 1).

Samples which were analyzed by capillary gel electrophoresis were collected from the HPLC and three 5-μl aliquots were dialyzed on a 0.025-μm membrane for 45 min over distilled water. This dialysate was injected electrokinetically into a gel-filled capillary and the DNA fragments were resolved using an applied field of 600 V/cm.

3. Results and discussion

The extraction, elution and quantitation of SODN DNA in human serum and urine was accomplished in 5 min using anion-exchange HPLC for on-line sample clean-up and analysis. The linearity, recovery, accuracy and precision of extraction and detection was determined. The chromatographic and electrophoretic profile of extracted DNA is shown.

Most molecular biology protocols for extraction of DNA from serum and other biofluids involve a proteinase K digestion step in which DNA-protein binding is disrupted through protein digestion [7]. We have used this approach to accurately quantify serum levels of antisense DNA at levels as low as 200 ng/ml [3]. However, this approach is time consuming and generates approximately 5 ml of toxic solvents (*i.e.* phenol,

Table 1
Data from interlaboratory collaboration

Sample identification	Actual conc. (ppm)	French result (ppm)	R.E.%	Hybridon result (ppm)	R.E.%
Spike 1	8.50	8.11	−4.6	8.14	−4.2
Spike 2	2.00	1.94	−3.0	1.94	−3.0
Spike 3	4.50	4.01	−10.9	4.27	−5.1
Spike 4	0.50	0.45	−10.0	0.41	−18.0

chloroform and *sec.*-butanol) per sample. We investigated the binding of our 25-mer antisense phosphorothioate oligomer (SODN) to serum proteins using size exclusion chromatography. We found no formation of DNA-protein adducts under conditions which we had shown could accurately monitor the adduct formation of the well known [8] *Escherichia coli* single-stranded DNA binding protein with phosphodiester $pd(T)_{20}$. This indicated that a protein digestion step was not necessary for this particular oligonucleotide and a direct extraction from serum should be possible. A phenol–chloroform extraction of the serum in the absence of protein digestion resulted in very poor recovery of the DNA. This presumably was a result of occlusion of the DNA in the denatured proteins. Thus, a non-denaturing extraction was necessary to achieve high recovery of DNA from the serum proteins. Because anion-exchange chromatography is known to *not* denature proteins, direct injection of raw serum, extraction of DNA from the protein matrix, and quantification of SODN by UV absorption should be possible. The high affinity of SODN for anion-exchange resins makes this assay not only quite specific, but very sensitive, since large volumes of sample can be injected with pre-column concentration and fast gradient elution producing very narrow peaks.

3.1. Method optimization

Elution solvent

The elution solvent in our previous work contained 30% formamide as a denaturant and to maintain the solubility of the phenol–chloroform extracted DNA. In our present work, all organic solvents were removed from the mobile phase since even small amounts caused protein precipitation and fouling of the analytical column. The mobilization salt was lithium bromide which was used because it eluted the SODN in very narrow peaks [3,9]. The pH of the mobile phase was buffered with Tris at pH 7.0 to reduce the negative charge on the serum proteins. EDTA (1 m*M*) was added to reduce heavy-metal contamination of the anion-exchange resin. Contamination of the system with Fe^{2+} is believed to cause partial oxidation of the phosphorothioate to phosphodiester [3]. Phosphodiester DNA has a much lower affinity for anion-exchange resins, thus the extracted DNA eluted over a broader salt concentration seriously increasing the detection limit.

3.2. Validation

Linearity

The UV response of SODN spiked into human serum was found to be a linear function of concentration from the detection limit (*ca.* 50 ppb) to 200 ppm. The detection limit was defined at a signal-to-noise ratio of 4. While the UV response was linear above 20 ppm, chromatographic efficiency began to diminish. Due to the small internal diameter of the column and the large sample volumes, the peak shapes became non-gaussian. Thus, samples that were above 20 ppm were diluted to between 1 and 5 ppm with 25 m*M* Tris buffer and reinjected.

Recovery

The recovery of SODN from human serum and urine was addressed by analyzing calibration curves prepared in serum, urine and buffer. Initial studies were performed at 40°C with the mobile phase buffered to pH 8. However, these conditions led to only 75–80% recovery of the DNA from the urine and serum as assessed by the ratio of the slopes of the calibration curves. Decreasing the pH to 7.0 and increasing the temperature to 50°C increased the lifetime of the column, sharpened the peak profile and increased the percent recovery to nearly 100%. Under these conditions, the calibration curves of the SODN analyzed in serum and buffer were coincident (Fig. 1).

The recovery of the analyte from the cellulose triacetate syringe tip filters was assessed by comparing the peak area of a 1-ppm standard before and after passing through the filter. The peak area remained unchanged indicating no loss of DNA to the filter.

The recovery for off-line extraction using solid-phase extraction was worse than the on-line

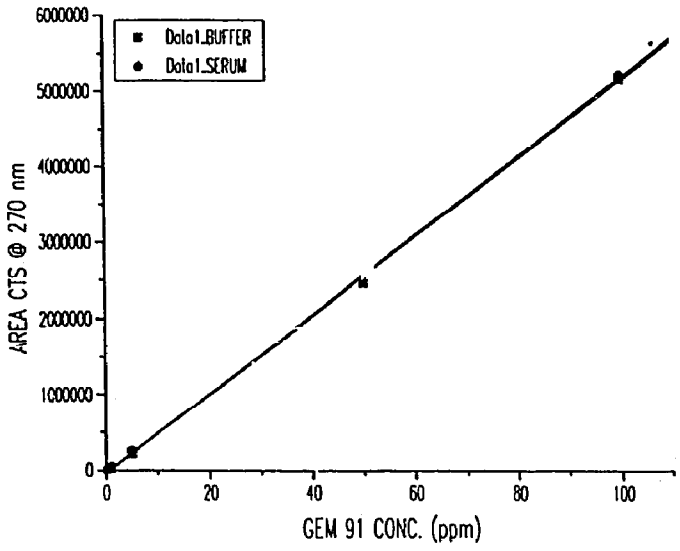

Fig. 1. Recovery *vs.* Matrix composition. A 25-mer SODN calibration curve was prepared in Tris buffer and human serum. The samples were analyzed and the peak area was plotted *vs.* the concentration in ppm. The curves are virtually coincident indicating equal recovery from buffer and serum.

method for several reasons. First the stationary phase for the on-line extraction of DNA from serum was a non-porous polymer based resin while the off-line extractions used porous silica-based anion-exchange cartridges. We have observed that porous silica-based strong anion-exchange HPLC columns exhibit very poor recovery of phosphorothioate DNA [9]. In addition, the inability to work at elevated temperatures decreased the efficiency of the off-line separation causing the DNA to elute from the cartridge as broad concentration zones. The addition of 20% ACN to the 2 *M* LiBr elution solvent increased the recovery to approximately 75–80%. This indicates a mixed-mode interaction (hydrophobic and ion-exchange) was occurring on the silica phase. The use of a fraction collector attached to the HPLC was found to be preferable to the off-line solid-phase extraction method since the recovery was higher, no organic solvents were necessary and the whole system could be automated.

Precision

The injection-to-injection precision was assessed by comparing the peaks areas of the standards over the course of an analysis. Peak area variations of less than 10% were considered acceptable. The criteria for system suitability was a correlation coefficient of no less than 0.99. The retention time during an analysis should not deviate more than 0.18 min which was equivalent to a relative error of 5% of the 2.7-min elution time. In practice this was met even with a change of column or mobile phase. This was due to the steepness of the gradient which minimized any small variations in column and mobile phase preparation. All of the data points for the standard injections over one week's worth of analyses ($n = 88$) were combined to yield a linear regression equation: $y = 47\,112x + 3449$, $r = 0.9975$. Thus, the day-to-day reproducibility of the calibration curve was excellent.

Accuracy

The standard calibration curve was prepared each day in the matrix of interest and was reanalyzed after every 10–12 sample injections. In this way, the system suitability was assessed often and any changes in recovery of the SODN from the biofluid would be immediately apparent from changes in the standard response.

As a test of accuracy and analyst bias, a double-blind spiked interlaboratory collaboration was performed in which the concentration of SODN spiked into human serum was determined. Four samples were prepared and stored at 4°C for one week. The samples were then shipped to France (at 0–4°C) for analysis using a similar HPLC system and columns which were hand-packed in our laboratory. The data were compared by a third party at our company and the results are shown in Table 1. The data indicate that a reasonably effective transfer of the method from our laboratory had been made. Deviations from the actual concentration spiked into the matrix were highest for the lowest concentration as expected.

3.3. Applications

Analysis of samples

The chromatograms of SODNs in urine and serum are virtually identical with respect to

recovery, retention time and peak shape. The chromatographic profiles were even quite similar despite the differences in the two matrices. This is due to the harsh conditions which are required to elute phosphorothioate DNA. There is virtually nothing in any of the matrices tested that interferes with the retention window of 25-mer phosphorothioate DNA. The slight broadness of the SODN extracted from a 6-month-old standard in serum was due to enzymatic degradation of the parent oligonucleotide (Fig. 2B). This enzymatic degradation was seen more clearly when the SODN fraction was collected from the HPLC, dialyzed and analyzed by capillary gel electrophoresis (Fig. 3B). This degradation was not a function of sample manipulation following fraction collection. This was proven by collecting the peak from a fresh standard and using the same technique for analysis by CE (Fig. 3C). No degradation was observed in the fresh sample. In addition, analysis of 100-ppm standards prepared in urine and buffer which were stored at 4°C for six months did not show the same profile of enzymatic degradation. Thus, combination of both techniques gives a quantitative answer for the amount of SODN present in the biofluid and a method to determine the level of degradation.

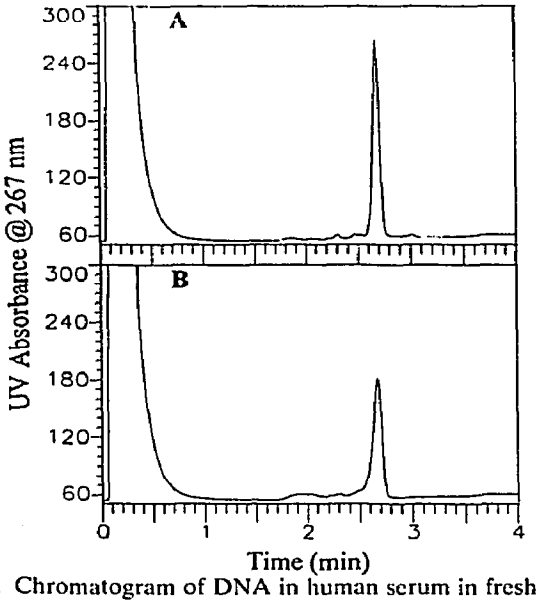

Fig. 2. Chromatogram of DNA in human serum in fresh and old standards. (A) 25-mer Phosphorothioate was spiked into human serum at a level of 100 ppm. The sample was diluted 5 × with buffer and a 100-μl aliquot was injected. (B) A sample that was prepared in the same manner and stored at 4°C for 6 months was analyzed as above. The increased bandwidth of the peak is due to the heterogeneity of the phosphorothioate DNA. Conditions: 20 × 2 mm I.D. Nucleopak PA-100 column. The salt gradient was 250 m*M* LiBr to 2000 m*M* LiBr over 2 min at 0.7 ml/min (50°C). The DNA was monitored at 267 nm.

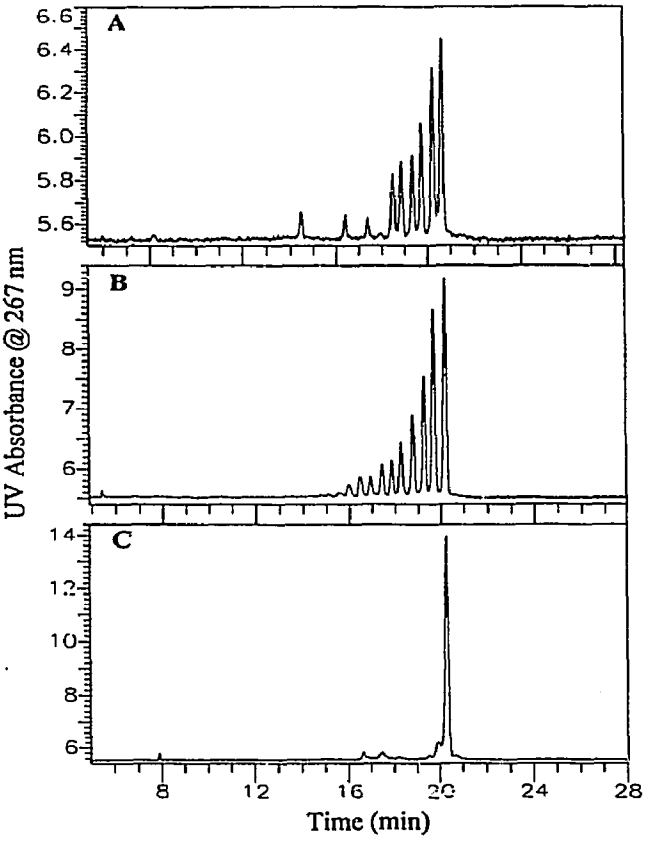

Fig. 3. Electropherogram of DNA extracted from human serum. (A) A mixture of 20-25-mer SODN standards was resolved under a field of 600 V/cm. (B) The sample that was stored for 6 months was collected and placed on a dialysis membrane over distilled water. After desalting, the sample was injected electrokinetically as above for the standards. (C) A freshly prepared sample was treated as above and analyzed. Conditions: 18% (w/v) acrylamide–30% (v/v) formamide–7 *M* urea–100 m*M* TBE buffer in a 75-μm capillary at 600 V/cm. The DNA was monitored at 267 nm.

Interferences

Even under optimal conditions anion-exchange HPLC does not have the resolving power to determine the ratio of 23, 24 and 25-mer present in the samples. Again this is unique to

phosphorothioates and is due to the strong secondary structure and the strength of the interaction of the SODNs with anion-exchange stationary phases. Using fast gradient conditions and very small columns, we did not expect to resolve the 25-mer SODN from the shorter fragments. The relative ratio of the oligomers could be clearly determined from capillary electrophoresis. No other compounds present in biological matrices have been found to interfere with the analysis.

4. Conclusions

A simple bioassay is presented for analysis of phosphorothioate DNA in various biological matrices. The method requires minimal sample handling and can analyze a sample every 5 min. Over one hundred 100-μl injections could be made without a pressure increase and concomitant column failure. The linear dynamic range of this method ranges from 200 ppm to 100 ppb with a detection limit of approximately 50 ppb. The combination of fast anion-exchange chromatography and capillary gel electrophoresis yields information on the quantity and character of phosphorothioate DNA extracted from a biological matrix.

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References

[1] J.C. Bigelow, L.R. Chrin, L.A. Mathews and J.J. McCormack, *J. Chromatogr.*, 533 (1990) 133.
[2] S. Agrawal, J. Temsamani and J.-Y. Tang, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 7595.
[3] A.J. Bourque and A.S. Cohen, *J. Chromatogr.*, 617 (1993) 43.
[4] A. Pompon, I. Lefebvre and J.-L. Imbach, *Biochem. Pharmacol.*, 43 (1992) 1769.
[5] A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith and B.L. Karger, *Natl. Acad. Sci. USA*, 85 (1988) 9660.
[6] D.N. Heiger, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.
[7] M. Gross-Bellard, P. Oudet and P. Chambon, *Eur. J. Biochem.*, 36 (1973) 32.
[8] U. Curth, J. Greipel, C. Urbanke and G. Maass, *Biochemistry*, 32 (1993) 2585.
[9] A.S. Cohen, M. Vilenchik, J.L. Dudley, M.W. Gemborys and A.J. Bourque, *J. Chromatogr.*, 638 (1993) 293.