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Stability measurement of oligonucleotides in serum samples using capillary electrophoresis

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Abstract

An in vitro stability study of unmodified and modified antisense oligonucleotides in human serum was performed with a previously developed capillary electrophoretic method using either micellar solution or entangled polymer solution depending on the oligonucleotide length to be separated. A method has been devised and validated for the extraction of oligonucleotides from serum using anion-exchange centrifugal filter units. The extracted samples were desalted by a drop dialysis method. The serum half-lives and the degradation patterns of unmodified and modified oligonucleotides are compared. The modified oligonucleotide used in this study is protected from exonuclease activity present in human serum by terminal 1,3-propanediol modification. © 1997 Elsevier Science B.V.

Keywords: Oligonucleotides

1. Introduction

Oligonucleotides which bind to complementary sequences in DNA, pre-mRNA or mRNA (so called antisense) have been shown to effectively inhibit the expression of the targeted gene [1]. These compounds are generally 12–30 bases long and can be used as potential therapeutic agents for the treatment of cancer and viral infections. Several oligonucleotides are now in clinical trials stage. Among others, the stability of these compounds against various endo- and exonucleases in biological media is a critical question in drug development. The biological instability of unmodified oligonucleotides can be overcome by using DNA analogues with modifications on either the phosphodiester linkage, the sugar or the base. The most extensively evaluated oligonucleotides are the first generation analogues, in particular, phosphorothioates [2–5], methylphosphonates [2,6], phosphoramidates [7] and phosphortriesters [8]. All of these derivatives have been shown to possess an increased resistance towards nucleases, but most of them displayed a lower binding to the target. An increased stability and binding affinity was observed, when both base and sugar modifications were introduced [9]. In sugar modifications alone, the use of α-oligonucleotides [6,10], oligonucleotides containing either α-L or β-L nucleotides [11] and oligonucleotides containing modified nucleosides were shown to demonstrate enhanced enzymatic stability [12,13]. The modified oligonucleotides used in this study involve a modification at

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the terminal 3′-nucleotide to block exonucleolytic degradation [14]. Previously, it has been proven consistently that the presence of 3′-modifications provides protection from degradation by 3′-exonuclease [4,5,15,16].

In order to perform the stability study of these antisense oligonucleotides, an automated, reliable and reproducible analytical method is required. A previously used electrophoretic separation and autoradiography technique for monitoring the degradation of ^{32}P-labeled oligonucleotides has several disadvantages [2,8]. Liquid chromatography (LC) requires large samples and shows inferior efficiency when compared to capillary gel electrophoresis (CGE) [17]. CGE employing cross-linked polyacrylamide gels is widely used for analyzing oligonucleotides [18]. However, a problem is encountered in the performance of these gel filled capillaries as they degrade over time, and in practice their lifetime is very limited. This paper describes the application of a previously developed capillary electrophoresis (CE) method using either entangled polymer solution or micellar electrokinetic capillary chromatography (MECC) for in vitro stability measurements of unmodified and modified antisense oligonucleotides against serum enzymes. It has been shown previously that unmodified oligonucleotides are rapidly degraded, primarily by 3′-exonuclease in serum [8]. It was also mentioned that the major site of oligonucleotides degradation lies outside the cell in serum or whole blood [19]. Previously obtained enzymatic stability data are incomplete because in most cases only purified enzymes were used and the stability observed in the presence of purified nucleases cannot be extrapolated to sera [10]. The effect of handling of the serum on the enzymatic stability is also evaluated as it has been shown that handling can have some effect on the enzymatic activity of serum containing media [6].

For sample clean-up, very few methods are described in literature to quantitate low levels of oligonucleotides in biological fluids such as serum [20]. Most methods require a significant amount of sample handling which includes time consuming protein digestion, phenol–chloroform extraction and ethanol precipitation of the extracted DNA. This classical methodology of phenol–chloroform extraction and ethanol precipitation for extraction of oligonucleotides present in serum is difficult to quantify, and hence subject to large errors. Bourque and Cohen [21] devised and validated an extraction method for the analysis of phosphorothioates from serum and urine. Their method consisted of a protein digestion step, a phenol–chloroform extraction, two 2-methyl-1-propanol and one diethyl ether extraction and was generated to overcome the non-quantifiable nature of ethanol precipitation, but it still remained a relatively laborious procedure. Therefore, to increase sample throughput and to reduce the amount of sample handling, an LC sample clean-up method as described by Pompon et al. [17] was slightly modified [22]. In this protocol an LC system is equipped with a switching valve coupled to an internal surface reversed-phase pre-column. Our work likewise describes the development of a new extraction method using anion-exchange centrifugal filter units for the extraction of oligonucleotides from serum samples.

2. Experimental

2.1. Chemicals

Oligonucleotide samples $p(dA)_{21}$, $(dA)_{21}$ and $(dA)_{25}$ [the latter two and orange G were used as internal standards (I.S.s] were purchased from Pharmacia Biotech (Roosendaal, Netherlands). Unmodified oligonucleotides (GS 1153, GS 1184) and a modified oligonucleotide (GS 1156) were assembled on a ABI 381 A DNA synthesizer following standard phosphoramidite chemistry [14] (for sequences and structures, see Fig. 1). All samples were stored in the freezer (−28°C). Frozen human serum and orange G were bought from Sigma–Aldrich (Bornem, Belgium). Hydroxyethyl cellulose (HEC EP09, >86.2% purity) was kindly donated by Union Carbide (Antwerp, Belgium). All other reagents used were of analytical grade. Tris [tris(hydroxymethyl)aminomethane], ammonium acetate and sodium dodecyl sulfate (SDS) were obtained from Acros Chimica (Geel, Belgium). Taps [N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] and urea were purchased from ICN Biomedicals (Aurora, OH, USA) and UCB (Brussels, Belgium), respectively. Sodium chloride was obtained from BDH (Poole, UK). Milli-Q water (Millipore, Milford, MA, USA) was used

ON	Sequence 5' to 3'	X (3'-substituent)
$p(dA)_{21}$ (21-mer)	AAAAAAAAAAAAAAAAAAAAA-X	-OH
GS 1153 (12-mer)	CAC CGA CGG CGC-X	-OH
GS 1156 (12-mer)	CAC CGA CGG CGC-X	$-O-PO_2-O-(CH_2)_3-OH$
GS 1184 (12-mer)	GAC ATC ATC CGC-X	-OH

Fig. 1. Structure and sequences of unmodified and modified oligonucleotide analogues.

throughout. Stock solutions of oligonucleotides samples were prepared in Milli-Q water as follows: $p(dA)_{21}$ 0.27 mg/ml, $(dA)_{21}$ 0.62 mg/ml, $(dA)_{25}$ 0.25 mg/ml, GS 1153 0.15 μg/ml and GS 1156 0.25 μg/ml.

2.2. Instruments

CE separation was performed on Spectra PHORESIS 1000 (Thermo Separation Products, Fremont, CA, USA) which was driven by CE software (version 3.01) operating under IBM OS/2TM (version 1.2). CE separation in entangled polymer solutions was carried out using a J&W DB-17 capillary (50% methyl silicone, 50% phenyl silicone coating) [44 cm (36 cm effective length)×100 μm I.D.], which was purchased from Alltech (Laarne, Belgium). The running buffer consisting of 20 m*M* Taps, 4% HEC EP09 and 7 *M* urea, was prepared as follows. 20 ml of water at 50°C was added to HEC while stirring. After obtaining a clear solution, 121.5 mg Taps was added (20 m*M*) and mixed. Then 10.5 g urea was added (7 *M*) and the mixture was stirred for a further 5–10 min. Finally the pH was adjusted to 7.0 with solid Tris and the final volume was made up to 25 ml with water [23].

For MECC, a fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA) of 44 cm (36 cm effective length)×50 μm I.D. was used. The running buffer used here contained 20 m*M* Taps, 70 m*M* SDS and 7 *M* urea (pH 7.7 adjusted with solid Tris) [24].

For extraction of oligonucleotides with the ultrafree-MC DEAE anion-exchange membrane (Millipore), a microcentrifuge (Eppendorf, 5415 C, Hamburg, Germany) was used. The following buffers were used for extraction purposes. Buffer A: 0.25 *M* (for 12-mer) or 0.45 *M* (for 21-mer) ammonium acetate, pH 7.0. Buffer B: 20 m*M* Tris and 1 *M* NaCl (pH 9.0 adjusted with HCl).

The extracted oligonucleotides were desalted by using membrane filters, composed of a mixture of cellulose acetate and cellulose nitrate, 0.025 μm (Millipore).

3. Results and discussion

3.1. Extraction protocol

Based on anion-exchange chromatography, we used ultrafree-MC DEAE (diethylaminoethyl) centrifugal filter units which incorporate a DEAE derivatised cellulose membrane in an insert to facilitate centrifugal anion-exchange separation. Negatively charged molecules such as oligonucleotides present in serum will bind to the positively charged DEAE groups on the membrane, while positively charged and uncharged molecules pass through to the microfuge collection tube. Subsequently, the bound proteins are washed away with loading buffer followed by elution of oligonucleotides with a buffer of stronger ionic strength.

The detailed extraction protocol used for the extraction of oligonucleotide from the serum is described below.

(1) Membrane preparation: to prepare the membrane surface, 0.4 ml of buffer A was added to the insert and the device was spun at 5000 *g* for 5 min. (2) Sample loading: 3 *M* ammonium acetate was added to 0.2 ml of serum containing 20 μl of oligonucleotide sample to obtain a concentration of 0.25 or 0.45 *M* ammonium acetate. The mixture was brought in the filter unit and centrifuged at 8000 *g* for 5 min. The oligonucleotides adsorb on the membrane and most of the serum proteins were

eluted. (3) Washing: to wash remaining serum proteins from the membrane, 0.4 ml of buffer A was added and centrifuged at 8000 *g* for 5 min. (4) 2nd washing: same as step 3. (5) Elution: to elute oligonucleotides, 0.16 ml of buffer B containing dA_{25} or dA_{21}, used as I.S., was added and centrifuged at 5000 *g* for 5 min.

To each filtrate from steps 2 to 5 were added four equivalent volumes of ice-cold acetone to test for the presence of proteins. The filtrate from steps 2 to 3 showed a positive test for proteins, while no precipitation of proteins was seen in filtrate from steps 4 to 5.

I.S. was dissolved in the elution buffer to prevent contact with serum enzymes, thus avoiding possible degradation. Therefore, addition of an I.S. will increase the precision of quantitation by accounting for the variation in the final extracted volumes and in the desalting procedure and for variation in sample injection.

The elution buffer was adjusted to pH 9.0 in view of the improved extraction recovery and a good buffering capacity of Tris at this pH.

3.2. Desalting

A particular problem with all forms of electrophoresis is the effect of the ionic strength of the sample. A phenomenon of sample "stacking" is well known in CE, where the sample ions are stacked together in a narrow sample zone due to the smaller ionic strength of the sample solution. Very often, however, the analytes are extracted from biosamples with buffers containing higher concentrations of salt.

The Millipore drop dialysis method was used to desalt samples with high ionic strength. About 80 μl of sample solution was placed on a 25 mm diameter membrane, which was placed on top of a deionized water layer contained in a petri dish. After about 45 min, the sample was recovered by micropipette and injected into the CE system. Electropherograms are shown in Fig. 2 (A and B). The comparison revealed that the peaks were sharper (high efficiency) and well shaped in Fig. 2B. Therefore the desalting method used here is an effective procedure to desalt samples of higher ionic strength.

In the extraction procedure, oligonucleotide samples were eluted with buffer B containing 1 *M* NaCl instead of 1 *M* ammonium acetate, as the NaCl salt was more readily dialyzed. This can be explained by the larger size of the ammonium acetate ions [25]. Indeed, elution with buffer B containing 1 *M* ammonium acetate followed by desalting, produced broad peaks on the electropherograms.

3.3. Recovery

The efficiency of the extraction procedure was studied for two different lengths of oligonucleotides. Recovery of the model compound $p(dA)_{21}$ and of the antisense oligonucleotide (GS 1153, 12-mer) was studied from spiked serum sample. This revealed that the extraction method had to be adapted to the size of the respective oligonucleotides. For the 12-mer, the strength of buffer A was decreased to 0.25 *M*

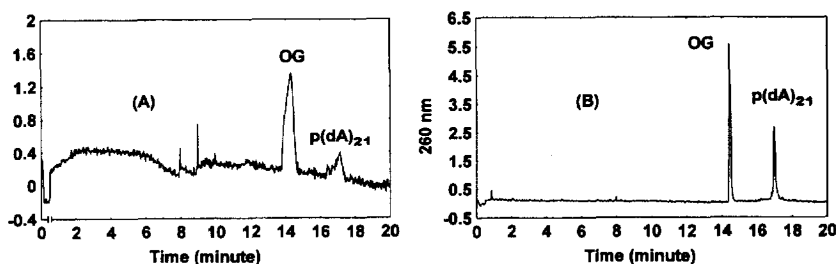

Fig. 2. The effect of the desalting procedure in preventing peak broadening of $p(dA)_{21}$, which was extracted from serum and injected as such (A) and after desalting (B). Electrolyte: 20 m*M* Taps, 4% HEC EP09 and 7 *M* urea (pH 7 adjusted with Tris). Capillary: J&W DB-17. Temperature: 25°C. Voltage: 12 kV at reversed polarity. Injection: hydrodynamic (30 s). Detection: UV at 260 nm. OG=orange G, used as I.S.

ammonium acetate pH 7.0 (for better extraction recovery). A 200 μl volume of blank serum was spiked with 20 μl of sample solution. The recovery was measured by comparing the response of extracted spiked samples with the response of reference solutions in water. The recoveries of $p(dA)_{21}$ and GS 1153 were 97% (R.S.D. = 1.6, $n=3$) and 86% (R.S.D. = 3.2, $n=4$), respectively. The relative standard deviation (R.S.D.) values were calculated after the entire process of extraction, desalting and analysis. The difference in the recoveries of the two compounds can be explained by a differences in the charge densities of the two compounds.

3.4. Incubation of homooligonucleotide [$p(dA)_{21}$] in human serum

To evaluate the degradation of the phosphodiester $p(dA)_{21}$ in human serum at 37°C, CE with an entangled polymer solution was chosen. This sample is degraded into $p(dA)_s$, shorter than 21 bases in length, which can be baseline resolved using CE with entangled polymer solution of HEC, as described previously [23]. The degradation profile of $p(dA)_{21}$ is presented in Fig. 3 and is in accordance with a 3′-end exonuclease activity.

3.5. Incubation of heterogeneous oligonucleotide in human serum

The 12-mer (GS 1153) was chosen for this study because of its excellent antisense properties in inhibiting the activated Ha-ras oncogene [26]. It was incubated in human serum at 37°C for different incubation times (0, 0.5, 1, 1.5 and 2 h). Oligonucleotides with base length of 12 or less are difficult to separate by CE using 4% HEC EP09 as entangled polymer solution. Therefore, MECC was used [24]. Fig. 4 shows the electropherograms of GS 1153 analyzed after the indicated incubation times. Due to the action of 3′-exonuclease, the formation of degradation products can be observed. The serum half-life of GS 1153 was calculated to be 1.4 h. The previous data for serum half-lives of unmodified oligonucleotides showed different results in human serum or heat-inactivated fetal calf serum (which was demonstrated to have comparable nuclease activity). In some cases, unmodified 12-mer [10] and 15-mer oligonucleotides [8,27] were reported with half-lives of 12, 7.7 and 5 min in human serum as determined in in vitro experiments, where in other reports, $t_{1/2}$ values of 0.5 [28], >2 [29] and ~4 h [2] were reported for 16-, 20- and 21-mer oligonucleotides. Therefore, comparison with our results seems difficult. The nature of the G-rich base sequence of GS 1153 may explain its better nuclease resistance ability [15]. Likewise, the partial self-complementarity of the studied sequence GS 1153 has to be considered. In addition, in vitro stability is always better than in vivo stability [28]. It should also be noticed that formation of N-1 increases with incubation time, while degradation of N-1 to N-2 is very slow. It can be concluded that enzymatic degradation of GS 1153 leads to the formation of a stable degradation product. The stability of the N-1 fragment can be very likely explained as a hairpin-like structure formation [28].

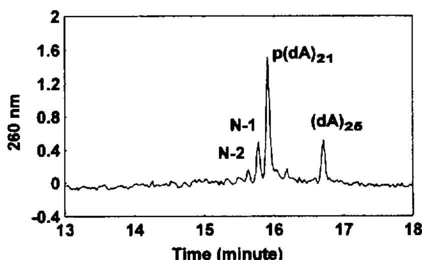

Fig. 3. Analysis of $p(dA)_{21}$ degradation in human serum (incubated at 37°C) after 2 h, using $(dA)_{25}$ as I.S. For CE conditions, see Fig. 2.

3.6. Incubation of modified antisense oligonucleotide in human serum

As shown in Fig. 4, GS 1153, as a regular phosphodiester oligonucleotide, is not suitable for therapeutic development, because of degradation by serum enzymes. For this reason, the 3′-end of the oligonucleotide was blocked by attachment of a polar moiety (1,3-propanediol) through a phosphodiester linkage (GS 1156) [14]. Fig. 5 represents the analyses of the sample, when incubated for 0, 4, 12 and 24 h in serum at 37°C. The calculated serum half-life of GS 1156 was 10.4 h. It can be concluded that 3′-end

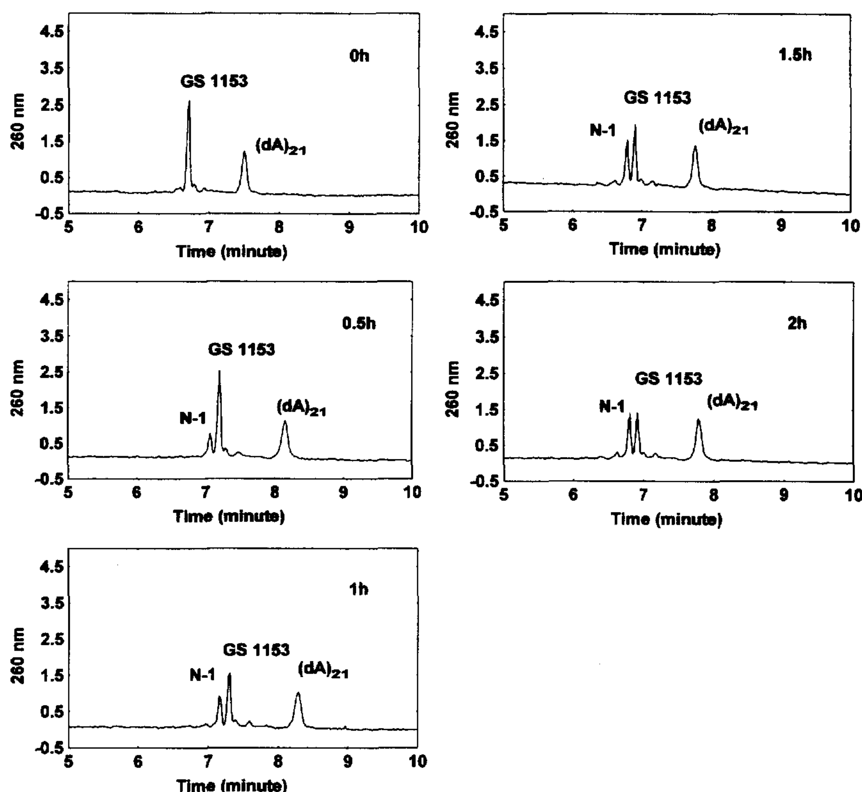

Fig. 4. Analysis of the unmodified 12-mer oligonucleotide GS 1153 following incubation in human serum at 37°C, using $(dA)_{21}$ as I.S. The electropherograms were obtained after the indicated incubation times. Electrolyte: 20 m*M* Taps, 70 m*M* SDS and 7 *M* urea (pH 7.7 adjusted with Tris). Capillary: fused-silica. Temperature: 25°C. Voltage: 23 kV. Injection: hydrodynamic (2 s). Detection: UV at 260 nm.

modification of the oligonucleotides leads to improved stability of the compound in serum.

3.7. Effect of hybridization and freeze–thaw cycle on enzymatic activity

As mentioned earlier, the serum half-life of GS 1153 is about 1.4 h. In order to check the influence of base sequence (GS 1153 has some duplex-forming probability), we compared the degradation rate of GS 1153 with GS 1184. Fig. 6 demonstrates that GS 1184 was degraded at a faster rate with a serum half-life of 0.8 h. Again this could point to partial self-hybridization of GS 1153 in serum, which therefore would afford a higher stability towards serum enzymes. It was demonstrated before that the 3′-exonuclease has a strong preference for single-stranded substrates [8].

In order to check the effect of freeze–thaw cycles, we compared two batches of human serum, one of which was thawed and frozen once while the other passed through more than five cycles. As can be seen in Fig. 7, the oligonucleotide degraded at faster rate in the first case. Therefore, it is important to evaluate the stability of oligonucleotides with serum batches which have not gone through several thawing–freezing cycles. The enzyme activity may have been destroyed in the second case due to multiple freezing and thawing cycles [6].

4. Conclusions

The previously developed CE methods were successfully applied for the in-vitro stability measurements of unmodified and modified antisense oligo-

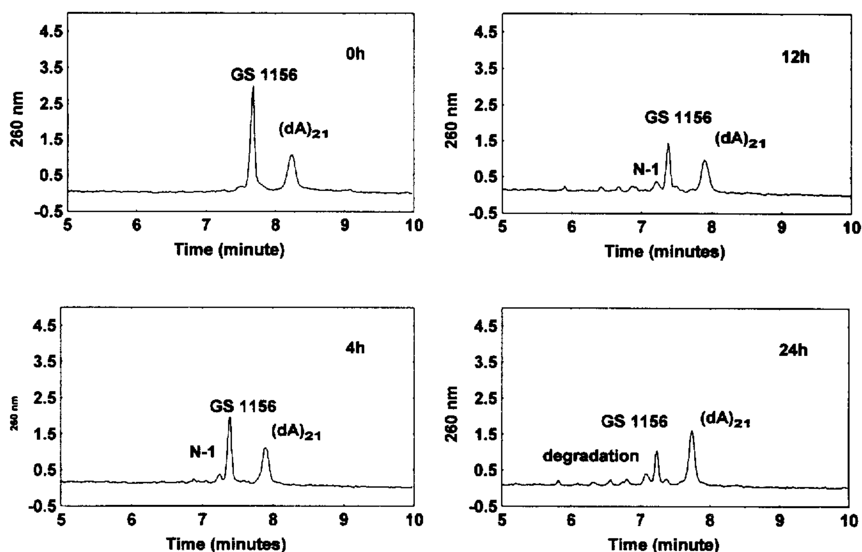

Fig. 5. Analysis of the modified 12-mer oligonucleotide GS 1156 after different incubation times in human serum at 37°C, using $(dA)_{21}$ as I.S. For CE conditions, see Fig. 4.

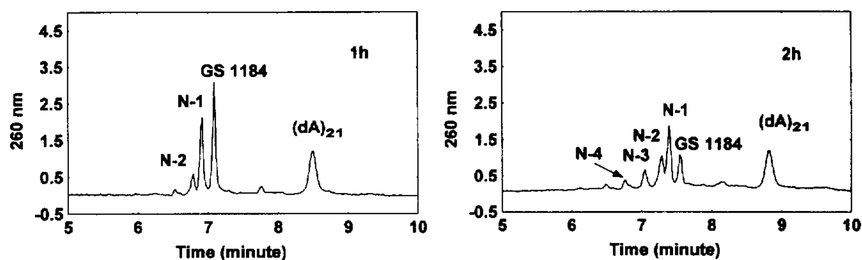

Fig. 6. Analysis of unmodified 12-mer oligonucleotide (GS 1184) after different incubation times in human serum at 37°C, using $(dA)_{21}$ as I.S. For CE conditions, see Fig. 4.

nucleotides in serum samples. The extraction method reported here selectively extracts the oligonucleotides from serum samples with good recovery. Oligonucleotides can be stabilized against enzymatic degradation by conjugating a 1,3-propane diol moiety at the 3′-end of the oligonucleotide and the

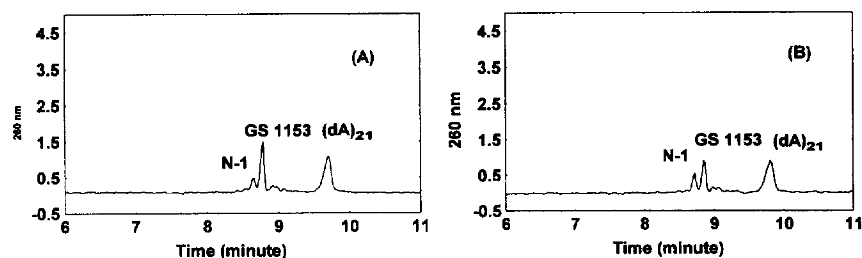

Fig. 7. The effect of serum handling on the degradation of GS 1153, using $(dA)_{21}$ as I.S. For CE conditions, see Fig. 4. (A) Human serum which was thawed–frozen several times and (B) serum which was thawed and frozen only once. The sample was incubated for 2 h at 37°C.

enzymatic stability of oligonucleotides is also dependent on the sequence hybridization ability and the number of freeze–thaw cycles of the serum.

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