ELSEVIER

Journal of Chromatography B, 669 (1995) 125–131

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Analysis of a ribonuclease H digestion of N3′ → P5′ phosphoramidate–RNA duplexes by capillary gel electrophoresis

Lawrence DeDionisio*, Sergei M. Gryaznov

Lynx Therapeutics Incorporated, 3832 Bay Center Place, Hayward, CA 94545, USA

Abstract

Phosphodiester oligonucleotides (ODNs) and their analogs are presently being investigated as potential antisense therapeutics in the treatment of viral infections and various forms of cancer. Here, we would like to report results from an investigation of activity for a ribonuclease H (RNase H) mediated RNA digestion assay in the duplexes formed by an ODN or the ODN analog, N3′ → P5′ phosphoramidate (3′-phosphoramidate), and complimentary RNA strands. Capillary gel electrophoresis (CGE) proved to be an effective method for determining RNA hydrolysis in the presence of RNase H. RNA and an ODN or RNA and a 3′-phosphoramidate were hybridized in a Tris-HCl, $MgCl_2$ buffer at room temperature (RT) and incubated with RNase H. Digestions were carried out at RT or at 37°C. Control samples were unhybridized RNA with RNase H, RNA without RNase H, and duplexes (RNA–ODN or 3′-phosphoramidate) without RNase H. All controls were incubated in Tris-HCl, $MgCl_2$ buffer, and sample aliquots were analyzed at various time intervals. A homodecamer, $(dT)_{10}$, was used as an internal standard to determine the relative migration time of the RNA strand. The final digestion products for the duplexes and the various controls were monitored by CGE. In addition, polyacrylamide gel electrophoresis (PAGE) was used in conjunction with Stains-All (staining) and a densitometric analysis to verify CGE results.

1. Introduction

It has been demonstrated that antisense oligonucleotides (oligos) inhibit viral growth [1] and can control gene expression in cell cultures [2]. Presently, antisense oligos in the form of synthetic DNA analogs are being investigated for pharmaceutical purposes in the treatment of viral infections and various forms of cancer [3–5]. The most common synthetic DNA analog utilized for these investigations is the phosphorothioate. CGE is often used to quantitatively determine the purity of synthetic phosphorothioates [6]. However, CGE has capabilities for a wider range of applications within the field of antisense therapeutics. We have recently begun to utilize CGE in studies involving DNA analogs and their susceptibility to nuclease digestion. Specifically, we present data here in which CGE was used to determine if duplexes formed between RNA and a new DNA analog, 3′-phosphoramidate (Fig. 1) serve as a substrate for RNase H.

The hydrolysis of RNA by RNase H after an antisense oligo has hybridized to the RNA target has been proposed as a reason for observed biological activity involving antisense oligos in

* Corresponding author.

SSDI 0378-4347(95)00153-0

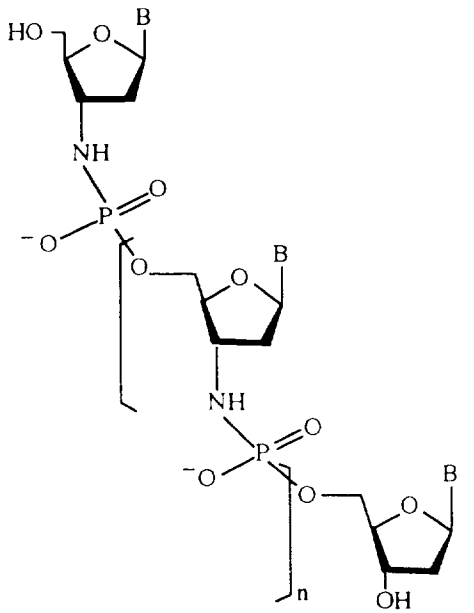

Fig. 1. Molecular structure of 3′-phosphoramidate. The -NH- group in the 3′-phosphoramidate backbone is substituted with an -O- atom in DNA. For the 3′-phosphoramidate used in this experiment, $n = 9$; thus, the full nucleotide length was $n + 2 = 11$. B represents one of 4 bases (A, G, C, or T) found in naturally occurring DNA. See Section 2 for base sequence.

cell culture experiments. Investigations into RNA hydrolysis by RNase H have been conducted utilizing ODNs as well as several different DNA analogs synthesized as complimentary strands for specific RNA targets [7–9]. These studies attempted to quantitate with slab gel electrophoresis the RNase H mediated hydrolysis of RNA. Our results here are an attempt to qualitatively illustrate the utility of CGE for studying antisense oligos and nuclease reactions in general with the focus on 3′-phosphoramidates and their ability to illicit an RNase H mediated hydrolysis of RNA.

3′-Phosphoramidates have been evaluated, and it was found that the duplexes they formed with DNA and RNA have higher melting temperatures (Tm) than duplexes formed by ODNs and complimentary DNA and RNA. They also formed stable triplexes with double-stranded DNA under conditions close to physiological [10]. They are therefore considered important as a potential oligonucleotide-based therapeutic; consequently, we are studying other properties of these compounds including their possible role in the RNase H mediated hydrolysis of RNA. CGE analysis revealed that RNA–3′-phosphoramidate duplexes do not appear to be substrates for RNase H. Thus in data presented here, hydrolysis of RNA, that has been duplexed with 3′-phosphoramidate, does not occur via RNase H cleavage.

2. Experimental

2.1. Synthesis of ODN, RNA, and 3′-phosphoramidates

The ODN (5′ CTT CTT CCT TA 3′) 1 and RNA (5′ AUA AGG AAG AAG C 3′) 2 oligos were synthesized on a Perkin Elmer, Applied Biosystems Division (ABI, Foster City, CA, USA) 380B DNA/RNA synthesizer. All synthesis reagents were purchased from ABI except for the following. RNA β-cyanoethyl phosphoramidite monomers were purchased from Pharmacia (Piscataway, NJ, USA), and capping reagent, phenoxyacetic anhydride, for RNA synthesis was obtained from TCI America (Portland, OR, USA). Standard β-cyanoethyl phosphoramidite synthesis cycles (1 μmol) were used for both ODN and RNA [11] syntheses. After synthesis, the RNA oligo was cleaved from the solid support with a mixture of conc. NH_4OH–ethanol (3:1, v/v). After heating at 55°C for 2 h, this solution was brought to dryness under a vacuum, and 1.0 *M* tetrabutylammonium fluoride in tetrahydrofuran (0.75 ml) purchased from Aldrich (Milwaukee, WI, USA), was added to remove silyl protecting groups. The sample was allowed to sit at RT overnight, and then concentrated to a solid residue under a vacuum. Sterile water (0.5 ml) was added, and the solution was desalted over a Sephadex G-25 (PD-10) column from Pharmacia. Ion-exchange chromatography was then carried out using a Dionex DX 300 (Sunnyvale, CA, USA) HPLC system and a Dionex nucleopac PA-100 (250 × 9 mm I.D.) column. Sample size was approximately 10 absorbance units (AU) in 1.0 ml. Chromatographic

conditions were as follows: buffer A was 0.01 *M* NaOH (pH 12.0); buffer B was 1.5 *M* NaCl and 0.01 *M* NaOH (pH 12.0); the flow-rate was 5.0 ml/min. A linear gradient of 1.0% buffer B/min was applied over 25 min, and fractions containing full-length RNA, monitored at 260 nm, were collected at 21.6% buffer B. The product was immediately acidified with 80% acetic acid to pH 5.0 and brought to dryness under a vacuum. The residue was dissolved in sterile water (1.0 ml) and desalted on a Sephadex G-25 column (Pharmacia). The ODN oligo was purified as previously described [12], and the 3′-phosphoramidate oligo 3 (see ODN sequence above) was synthesized and purified as previously described [10].

2.2. CGE and polyacrylamide gel electrophoresis

CGE was conducted as previously described [12,13]. In brief, either an ABI 270A or ABI 270A-HT instrument was used. Capillaries (100 μm I.D. × 375 μm O.D. × 50 cm; 20 cm to detector window) purchased from Polymicro Technologies (Phoenix, AZ, USA) were filled with ABI's Micro-Gel (10%) 35 m*M* Tris, 5.6 m*M* H_3BO_3, and ethylene glycol (EG, 15%), pH 9.0. Runs were conducted with a (−) polarity at a constant voltage of 22 kV. Injections were electrokinetic for 60.0 s (−15 kV); detection was set at 260 nm, and temperature was kept at 55°C. Sample concentrations [(AU)/ml at 260 nm] were pre-determined and set at 1.0.

Polyacrylamide gel electrophoresis (PAGE) analysis was conducted with a Hoefer (San Francisco, CA, USA) SE 600 electrophoresis unit. Gels (0.75 mm thick) were 20% acrylamide–bis-acrylamide (ratio 20:1), 0.06% w/v $(NH_4)_2S_2O_8$ and 0.06% v/v N,N,N′,N′-tetramethylethylenediamine in 7 *M* urea and 40 m*M* Tris-borate, pH 8.2. Runs were conducted at constant current (10 μA) for approximately 45 min, stained with Stains-All reagent purchased from Kodak (Rochester, NY, USA) and imaged on a Molecular Dynamics (Sunnyvale, CA, USA) densitometer. All electrophoresis reagents were purchased from Aldrich except EG which came from Fisher (Pittsburgh, PA, USA).

2.3. RNase H digestion assay

The RNA oligo 2 (1.0 AU/ml) and ODN oligo 1 (1.0 AU/ml) or 3′-phosphoramidate 3 (1.0 AU/ml) were incubated at either 37°C or at RT in a reaction mixture volume of 100 μl containing 10 m*M* Tris-HCl (pH 7.2), and 10 m*M* $MgCl_2$. *E. Coli* RNase H (1.1 unit) from Pharmacia was added, and CGE was used to analyze aliquots at 15 min, 30 min, 1 h, 3 h, and 24 h. For reactions incubated at 37°C, aliquots were also analyzed at 48 h and 96 h. Controls consisted of neat RNA oligo incubated with and without RNase H, and RNA–ODN or 3′-phosphoramidate duplexes incubated without RNase H. An internal standard [$(dT)_{10}$; 1.0 AU/ml] was used to determine relative migration times for the digestion assay containing the RNA–3′-phosphoramidate duplex. This homodecamer was synthesized and purified like the ODN strand 1 described above.

3. Results and discussion

3.1. RNase H digestion assay for the RNA–ODN duplex

In evaluating the RNA–3′-phosphoramidate duplex as a substrate for RNase H, it was necessary to determine sample conditions that were compatible with a CGE analysis without impeding possible enzymatic activity. Customarily, for CGE analysis of oligos, samples are dissolved in water or in solvents lower in ionic strength than the electrolyte in order to reduce zone broadening which can occur with UV detection [14]. Zone broadening decreases when the sample is dissolved in pure water; however, with the proper injection parameters, such as an increased voltage during the injection as well as increasing the injection time, peak broadening does not occur even when salts are present in the sample matrix [15]. With this in mind, we kept buffer concentrations as low as the digestion

assay would allow and increased the standard injection voltage from 8 to 15 kV and time from 5.0 to 60.0 s [13]. A control was then necessary to ensure that the selected buffer conditions would not be a factor in determining RNase H activity in the presence of the RNA–3′-phosphoramidate duplex. This control consisted of the RNA strand 2 hybridized to the ODN strand 1 with the same base sequence as the 3′-phosphoramidate 3.

In Fig. 2, a series of electropherograms illustrate the digestion of the RNA strand 2 over a period of three hours at RT in the presence of the ODN oligo 1 and RNase H. Calculating the ratio of the migration times between peaks corresponding to the ODN and RNA within each electropherogram served to correct for variations in migration times from run to run. This ratio can be defined as the relative migration time (RMT). At the start of the digestion (Fig. 2A), the faster moving peak, corresponding to the ODN, is seen at time (t) = 13.0 min while the RNA peak is at t = 14.9 min. The RMT (t_{RNA}/t_{ODN}) = 1.15; this ratio was constant throughout the course of the digestion. After 15 min (Fig. 2B), the RNA peak was observed to decrease significantly until at 3 h (Fig. 2D) it became undetectable. Consequently, the products of the digestion were indicated by an increase in the number and size of additional peaks over time (compare Fig. 2A to 2D). These additional peaks correspond to shorter segments of RNA as the RNA strand was hydrolyzed in the presence of complimentary DNA and RNase H. The fact that the RNA strand was hydrolyzed over a period of 3 h at approximately 21°C indicated that the enzyme was active under the reaction conditions we selected.

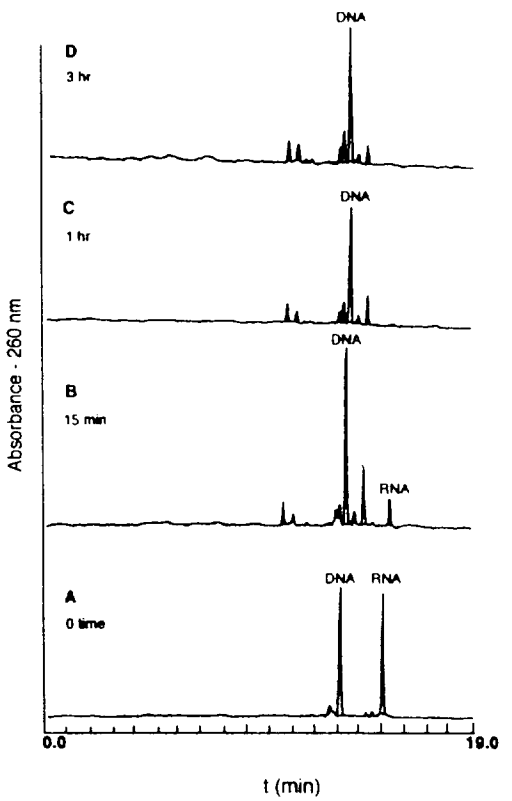

Fig. 2. CGE analysis of the RNase H digestion of the RNA ODN duplex. Sample (1.0 AU/ml) was buffered in 10 m*M* Tris-HCl (pH 7.2) and 10 m*M* $MgCl_2$. Electrophoresis was conducted with an electrokinetic injection of 15 kV for 60 s and a running voltage of 22 kV. Running buffer consisted of 35 m*M* Tris, 5.6 m*M* H_3BO_3, 15% EG, pH 9.0. Micro-Gel concentration was 10%. Sample was incubated at RT with 1.1 units of RNase H. Electropherograms were conducted at (A) 0 time, (B) 15 min, (C) 1 h, and (D) 3 h. A relative migration time or RMT (t_{RNA}/t_{DNA}) was used to identify the RNA peak within the appropriate electropherograms. A: RMT = 14.9/13.0 = 1.15; B: RMT = 15.3/13.3 = 1.15. DNA = ODN.

3.2. RNase H digestion assay of the 3′-phosphoramidate–RNA duplex

When the digestion was repeated using the 3′-phosphoramidate strand 3 as the template in place of the ODN strand 1, the starting compounds were not easily identified. It was necessary to utilize an internal standard in order to identify the RNA peak. Internal standards in conjunction with CGE have been demonstrated to be reliable and relatively precise for data analysis [16]. We routinely use an internal standard to check batch to batch consistency and efficiency of our gel-filled capillaries. A synthetic homodecamer, $(dT)_{10}$, is employed as the internal standard because its mass ratio (q/m) and thus its migration properties on a capillary gel are relatively equal to the oligos used for our experiments. Calculating an RMT by utilizing the

migration time of $(dT)_{10}$ as the reference, the RNA peak for this digestion assay was identified.

After the peak corresponding to RNA was identified within electropherograms containing the RNA–3′-phosphoramidate duplex, we performed the digestion assay with this duplex. We chose not to include the internal standard in the digestion assay since it was necessary to duplicate the experimental conditions of our control (Fig. 2). After 24 h at RT, the resulting electropherograms (data not shown) revealed that RNA hydrolysis was not occurring. At this point, we decided to conduct the assay at 37°C which would have accelerated any enzyme catalyzed hydrolysis.

Fig. 3 illustrates the results of the RNase H digestion assay conducted with the RNA–3′-phosphoramidate duplex at 37°C. After 24 h (Fig. 3B) the electrophoretic profile was relatively unchanged from the electropherogram obtained at the start of the assay (Fig. 3A). After 4 days at 37°C (Fig. 3C) sample degradation was evident by the increase in the number and size of faster migrating peaks; however, we determined that these degradation products were not due to the presence of RNase H. Controls, consisting of unhybridized RNA with RNase H, RNA without RNase H, and RNA–3′-phosphoramidate duplex without RNase H all contained similar degradation products after 4 days at 37°C. The increase in faster moving peaks in Fig. 3C, implicating a partial hydrolysis of the RNA strand, was not due to the presence of RNase H since similar results were obtained in controls where RNase H was not present.

3.3. PAGE analysis

PAGE analysis was also conducted on the reaction mixture at the final point (Fig. 4). Lane 1 contains the RNA–ODN duplex without RNase H while lane 2 contains the same duplex in the presence of RNase H. Lane 2 corresponds to the electropherogram of Fig. 2D. A band representing the full-length RNA strand was still detectable with PAGE while CGE was unable to detect any full-length RNA in this sample. Otherwise, PAGE analysis of the final results for

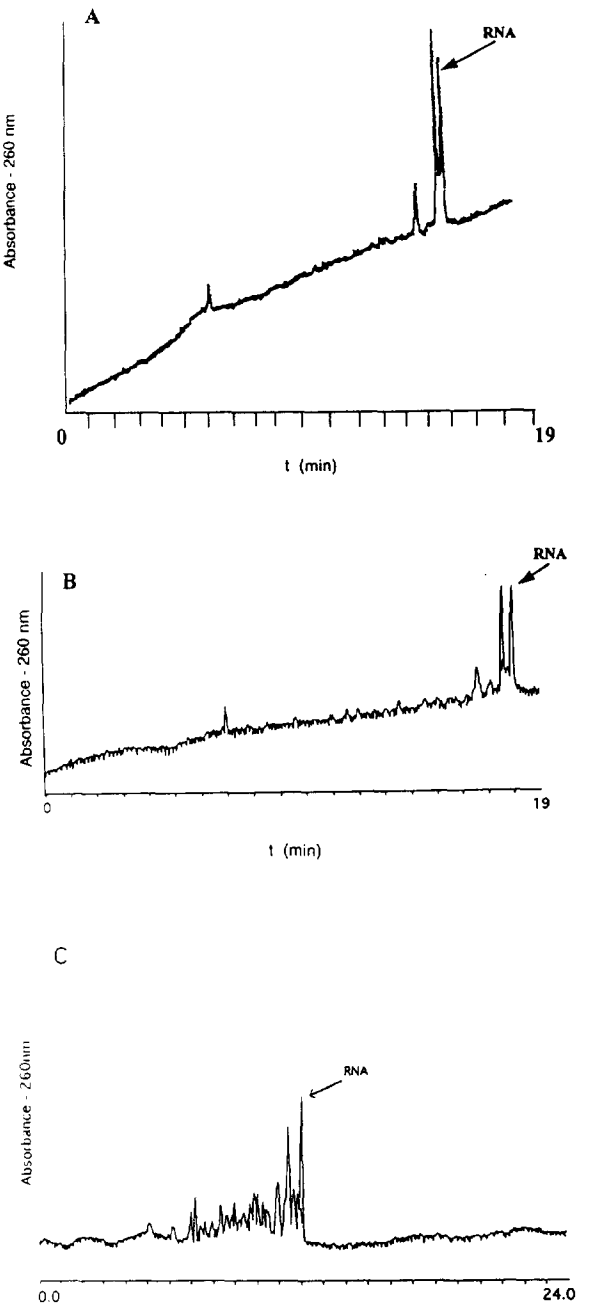

Fig. 3. Analysis of RNase H digestion assay of the RNA–3′-phosphoramidate duplex at time = (A) 0 min, (B) 24 h, and (C) 4 days. Reaction mixture consisted of RNA, 3′-phosphoramidate, and RNase H in 10 m*M* Tris-HCl (pH 7.2) and 10 m*M* $MgCl_2$. Sample matrix, gel parameters, running conditions for CGE, and RNase H concentration were the same as in Fig. 2 except that the digestion was conducted at 37°C.

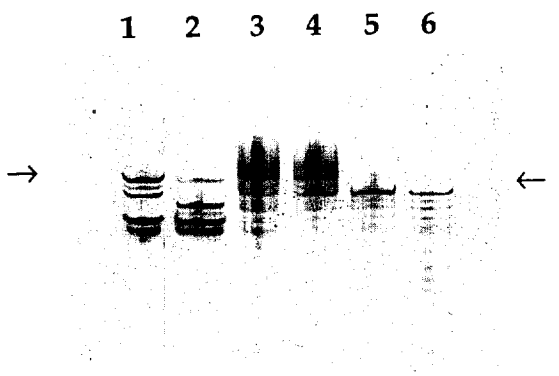

Fig. 4. PAGE analysis of final RNase H digestion products. Lane 1: RNA–ODN duplex incubated at RT without RNase H for 3 h. Lane 2: RNA–ODN duplex incubated at RT with RNase H for 3 h. Lane 3: RNA–3′-phosphoramidate duplex incubated at 37°C without RNase H for 4 days. Lane 4: RNA–3′-phosphoramidate duplex incubated at 37°C with RNase H for 4 days. Lane 5: RNA strand incubated at 37°C with RNase H for 4 days. Lane 6: RNA strand incubated at 37°C without RNase H for 4 days. Arrows indicate mobility of full-length RNA.

each of the digestion assays were comparable to the CGE data (see Fig. 2 and Fig. 3).

3.4. Advantages of CGE over conventional slab gel electrophoresis

The general advantages of CGE over slab gels lie in the durability of gel-filled capillaries and in the on-line detection system that most capillary electrophoresis units contain. The disadvantage of the slab-gel's requirement for a post-electrophoretic chemical staining is that different DNA analogs strain differently, i.e., 3′-phosphoramidates do not stain with the same efficiency as ODNs [10]. Differences in staining efficiency are not well understood at this time, and thus the accuracy of quantitative analysis utilizing PAGE–Stains-All in conjunction with a densitometer is questionable. Comparison studies between HPLC, PAGE, and CGE have been conducted, and it was found that Stains-All does not always bind to certain DNA analogs as a linear function of the analog's base length [17]. The on-line detection system utilized by CGE circumvents the necessity for post electrophoretic processing.

The other critical advantage of CGE over slab gel electrophoresis is the capacity for a gel-filled capillary to handle a larger number of samples than a typical PAGE gel which contains about 12 wells per gel. Our gel-filled capillaries are durable for up to 40 runs [12] which allowed us to use one capillary to analyze our digestion assay from start to finish (a 4 day time interval). It would have been necessary to pour at least three slab gels involving time-consuming sample preparation and post electrophoretic gel manipulation to obtain the data that was retrieved from one gel-filled capillary. We therefore ran only one PAGE gel on the last day of the experiment in order to verify the final CGE results.

A more specific advantage that CGE gave us for this experiment is that samples were injected directly from the reaction mixture. Sample preparation prior to an injection was unnecessary because the sample buffer (10 m*M* Tris-HCl; 10 m*M* $MgCl_2$) was lower in ionic strength than the running buffer (35 m*M* Tris; 5.6 m*M* H_3BO_3; 15% EG). Injecting directly from the reaction vial allowed continuous monitoring of the enzymatic assay without the inconvenience of aliquoting samples at pre-determined time intervals for the purpose of a slab gel analysis. Conventional PAGE or agarose gels require samples to be dissolved in a suitable denaturant like formamide; thus, evaporation of the reaction buffer, which will quench the enzyme reaction, must be carried out before the sample can be dissolved in the appropriate loading buffer. This introduces the possibility of sample contamination due to the ubiquitous nature of ribonucleases in general, which can hydrolyze RNA in an unpredictable manner. Therefore, sample handling, i.e., aliquoting from the reaction mixture into separate eppendorf tubes, should be avoided as much as possible. Finally, the ease that CGE provides for a qualitative study of an enzyme digestion assay like that presented here warrants the use of this electrophoretic tool for more extensive enzyme experiments which might involve kinetics and a quantitative approach to time-course studies.

4. Conclusions

We have demonstrated here that CGE is a useful tool in determining if an RNA–3′-phosphoramidate duplex is a substrate for RNase H digestion. Reaction conditions were carefully selected allowing us to directly analyze from the reaction mixture. Therefore, sample preparation was unnecessary prior to injection onto the gel capillary. To overcome factors within the sample matrix that would inhibit an injection, injection parameters were optimized. We have not attempted to present a quantitative analysis here, for at this stage in our study of the 3′-phosphoramidate analog, extensive quantitative studies are superfluous until it has been determined that these compounds provoke an antisense response in vivo. However, in lieu of the work that others have conducted in the antisense field [6,17], the nature of CGE renders a more quantitative approach to the susceptibility of RNA to hydrolysis via RNase H in the presence of a DNA analog.

We also found the RNA strand to be considerably stable. In dilute solutions (1.0 AU/ml) we expected the RNA strand to be susceptible to degradation from naturally occurring ribonucleases. However at RT, even after several days, very little RNA hydrolysis occurred. In conclusion, the results of this study indicate that CGE should prove a valuable tool for other experiments involving enzyme-mediated hydrolysis of RNA, DNA, or a DNA analog.

Acknowledgements

We would like to thank Drs. J.-K. Chen, D. Lloyd, and R. Schultz for support of this work.

References

[1] P.C. Zamecnik and M.L. Stephenson, *Proc. Natl. Acad. Sci. USA*, 75 (1978) 280–284.
[2] E.L. Wickstrom, T.A. Bacon, A. Gonzalez, D.L. Freeman, G.H. Lyman and E. Wickstrom, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 1028–1032.
[3] M. Matsukura, G. Zon, K. Shinozuka, M. Robert-Guruff, T. Shimada, C.A. Stein, H. Mitsuya, F. Wong-Staal, J.S. Cohen and S. Broder, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 4244–4248.
[4] S. Agrawal, T. Ikeuchi, D. Sun, P.S. Sarin, A. Konopka, J. Maizel and C. Zamecnik, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 7790–7794.
[5] L. Perlaky, Y. Saijo, R.K. Busch, C.F. Bennett, C.K. Mirabelli, S.T. Crooke and H. Busch, *Anti-Cancer Drug Design*, 8 (1993) 3–14.
[6] G.S. Srivatsa, M. Batt, J. Schuette, R.H. Carlson, J. Fitchett, C. Lee and D.L. Cole, *J. Chromatogr.*, 680 (1994) 469–477.
[7] P.J. Furdon, Z. Dominski and R. Kole, *Nucleic Acids Res.*, 17 (1989) 9193–9204.
[8] R.V. Giles and D.M. Tidd, *Anti-Cancer Drug Design*, 7 (1992) 37–48.
[9] G.D. Hoke, K. Draper, S.M. Freier, C. Gonzalez, V.B. Driver, M.C. Zounes and D.J. Ecker, *Nucleic Acids Res.*, 19 (1991) 5743–5748.
[10] S. Gryaznov and J. Chen, *J. Am. Chem. Soc.*, 116 (1994) 3134–3144.
[11] Applied Biosystems User Bulletin (1989) No. 53.
[12] L. DeDionisio, *Am. Lab.*, July (1994) 30–34.
[13] L. DeDionisio, *J. Chromatogr.*, 652 (1993) 101–108.
[14] A. Paulus and J.I. Ohms, *J. Chromatogr.*, 507 (1990) 113–123.
[15] J. Macek, U.R. Tjaden and J. van Der Greef, *J. Chromatogr.*, 545 (1991) 177–182.
[16] A. Guttman, R.J. Nelson and N. Cooke, *J. Chromatogr.*, 593 (1992) 297–303.
[17] A.S. Cohen, M. Valenchik, J.L. Dudley, M.W. Gemborys and A.J. Bourque, *J. Chromatogr.*, 638 (1993) 293–301.