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Stability measurements of antisense oligonucleotides by capillary gel electrophoresis

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Abstract

The approach of using antisense oligonucleotides as potential drugs is based on hybridization of a short chemically-modified oligonucleotide with complementary cellular DNA or RNA sequences. A critical question is the stability of chemically modified antisense oligonucleotides in cellular environments. In a model system, resistance against various nucleases was evaluated by capillary gel electrophoresis (CGE). For some of the samples, matrix assisted laser desorption and ionization mass spectrometry (MALDI-MS) was used as an additional analytical tool to perform stability measurements.

Using CGE, the enzymatic degradation of single nucleotides from the oligomer can be followed after different incubation times. 10% T polyacrylamide gels give baseline resolution for oligonucleotides ranging between 5 and 30 bases in length. The kinetic influence of a specific nuclease concentration and the antisense oligonucleotide structure on the cleavage reaction are discussed. Also, a simple desalting method to improve the injection efficiency and sensitivity of the method are described. Examples of measurements of chemically modified antisense 19-mers are presented.

1. Introduction

Short segments of single-stranded oligonucleotides, complementary to a specific gene, have received attention as potential therapeutic agents in recent years [1,2]. The idea behind these antisense or anti-gene agents is that they inhibit a specific gene expression, either in the nucleus by binding to double-stranded DNA and pre-mRNA or in the cytoplasm by binding to mRNA. In this way the production of a specific, harmful protein can be prevented. The main problems associated with a therapeutic development are the delivery of the antisense agents to the cells, uptake across the membrane [3,4], and the in vivo stability against nucleases [5–7]. In order to overcome these stability and uptake problems, several DNA analogues with backbone, desoxyribose, and base modifications have been described. One of the earliest backbone modifications is in the phosphorothioates with the substitution of a sulfur atom for a non-bridging oxygen atom in the phosphodiester [8]. Later, the replacement of the complete deoxyribose-phosphate backbone by an amide functional group [9], which renders so-called peptide nucleic acids (PNA), and mixed phosphodiester–

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amid bonds [10] are described. In addition to the backbone modifications, several ribose modifications show improved stability under physiological conditions compared with the phosphodiester bond.

The intensive research in the field of antisense oligonucleotides nowadays asks for a fast, reliable, highly-efficient analytical technique in order to perform purity and stability checks of these type of synthesized compounds.

Capillary gel electrophoresis (CGE) has proven to be a suitable analytical method with high resolution for the separation of modified and unmodified oligonucleotides, as demonstrated by several authors [11–14]. Cohen et al. [11,12] indicated that HPLC separations were inferior with respect to resolution compared CGE separations for the analysis of phosphorothioates and phosphodiesters.

Rose [13] studied the binding of peptide nucleic acids to their complementary oligonucleotide with CGE. He showed that CGE was useful in resolving the free and bound species which in turn also allowed the calculation of binding kinetics and the stoichiometry of binding.

The influence of gel concentration, pH and buffer additives, was proven to be critical in obtaining high resolution and gel-to-gel reproducibility for the analysis of phosphorothioates as investigated by DeDeionisio [14].

This paper describes the use of CGE for the stability measurements of antisense oligonucleotides. The influence of various endo- and exonucleases on the stability of unmodified and modified oligonucleotides will be discussed. In order to get a better insight on the cleavage reactions, some of the samples were also analyzed at various incubation times with matrix assisted laser desorption and ionization mass spectrometry (MALDI-MS). This technique allowed identification by molecular mass of the degradants formed.

The ultimate goal of analysing cell extracts was checked by injecting samples with high salt concentrations. It is well known in CGE (and in CE in general) that electrokinetic injection of samples with high conductivity results in a decreased amount of sample. This problem can be partly circumvented by a simple and rapid desalting procedure.

2. Experimental

2.1. Instrumentation

Capillary gel electrophoresis was carried out on a Hewlett-Packard ^{3D}Capillary Electrophoresis system (Waldbronn, Germany) using polyacrylamide gel-filled capillaries (10% T, 0% C), which were prepared in house according to a previously published method [15]. Data acquisition and data analysis were done with HP ^{3D}CE analysis software.

The separations were performed in 75 μm I.D. capillaries with 32 cm total length (24 cm effective length), using a separation voltage of −10 kV. The capillary was thermostated at 30.0°C and the sample tray at 24°C. The background electrolyte consisted of 100 m*M* Tris and 100 m*M* boric acid at pH 8.4. These conditions resulted in currents between 3 and 4 μA. Injection was done electrokinetically at the cathodic site at 5 kV for 5 s.

Micellar electrokinetic capillary chromatography (MEKC) separations were carried out using a Prince (Lauerlabs, Emmen, Netherlands) capillary electrophoresis system.

MALDI-MS measurements were recorded on a LDI-1700 instrument from Linear Scientific (Reno, NV, USA). The linear time-of-flight mass spectrometer was calibrated with a peptide standard mixture for conversion from flight times to molecular masses. All measurements were carried out in the negative ion mode, using dihydroxyacetophenone–diammoniumhydrogencitrate as a matrix [16].

2.2. Chemicals

$p(dT)_{20}$ and Orange G, which was used as an internal standard in some of the experiments, were obtained from Sigma (St. Louis, MO, USA). The other modified and unmodified oligonucleotides were synthesized in the Central Re-

search Laboratories of Ciba-Geigy or at ISIS (Carlsbad, CA, USA). The phosphodiesterase from *Crotalus durissus* (snake venom nuclease) and the endo/exonuclease Nuclease S1 were purchased from Boehringer (Mannheim, Germany).

Tris(hydroxymethyl)aminomethane (Tris), boric acid, sodium dodecysulfate (SDS), sodium dihydrogenphosphate, dihydroxyacetopheonone and diammonium hydrogencitrate were of analytical grade and obtained from Fluka (Buchs, Switzerland).

2.3. Methods

The general procedure for stability measurements of oligonucleotides involved mixing a small volume of an oligonucleotide solution (dissolved either in water or in buffer), typically between 10 and 20 μl, with 1 to 5 μl of a nuclease solution. Injections into the gel-filled capillary were performed directly from the incubation vial at regular time intervals.

For the MALDI-MS measurements 1.4 μl incubation solution (with the same composition as for the CGE measurements) was mixed with 1.4 μl matrix solution (a mixture of 10 m*M* 2,6-dihydroxyacetophenone and diammonium hydrogencitrate in acetonitrile). From this, 0.4 μl was placed onto the probe tip and rapidly dried with vacuum to avoid separation during the crystallization process. The complete sample preparation was typical and is described elsewhere [16].

The following procedure, called drop dialysis, was used for desalting samples of interest. About 10 μl reaction mixture were pipetted on a small Millipore filter with a pore size of 0.025 μm, which was placed on top of a water surface. The sample was desalted after approximately 30 min. The desalted aliquot was then put in a sample vial and injection was performed in the usual way.

3. Results and discussion

The mass-to-charge ratio of oligonucleotides decreases rapidly from 2 to 5 nucleotides and reaches a plateau above 10 nucleotides. It implies that separations based on a difference in charge can be carried out in free solution without a problem in case of oligonucleotides smaller than 10 bases in length. However, the electrophoretic mobility in free solution is almost constant for oligonucleotides with more than 10 bases. In the size range between ten and thirty bases, the sieving effect of gels becomes mandatory. This can be seen in Fig. 1A and B. Whereas in the electropherogram in Fig. 1A where the $p(dT)_{2-11}$ mixture is baseline resolved, the resolution for the $p(dA)_{12-18}$ mixture decreased considerably. In both examples SDS was used in order to improve resolution. The addition of Mg(II) or Zn(II) in millimolar concentrations to the background electrolyte, as suggested by Cohen et al. [17], resulted only in small improvements of the $p(dA)_{12-18}$ separation.

These results clearly demonstrate that the use of sieving gels (or suitable polymer solutions [18]) is necessary in order to separate on the basis of a difference in size, rather than on a difference in charge.

3.1. Incubation of $p(dT)_{20}$ with snake venom nuclease

A simple model system has been chosen to develop and evaluate a CGE protocol to study precise kinetics of the degradation of a specific oligonucleotide in various media. It consisted of phosphodiester $p(dT)_{20}$ as model compound which reacts with snake venom nuclease. $p(dT)_{20}$ was chosen for its commercial availability in a pure form and its well-defined reaction products [only p(dT)s, smaller than 20 bases in length], which can be baseline resolved with CGE. The exonuclease attacks exclusively at the 3′-end of an oligonucleotide. Immediately after vortex mixing the $p(dT)_{20}$ solution, dissolved in buffer and the snake venom nuclease solution, the first injection was performed. Consecutive injections were done automatically at regular time intervals. The $p(dT)_{20}$ concentration was $4 \cdot 10^{-3}$ μg/μl in 40 m*M* Tris–HCl, 4 m*M* $MgCl_2$, pH 9.0 buffer. A total reaction volume of about 20 μl was used.

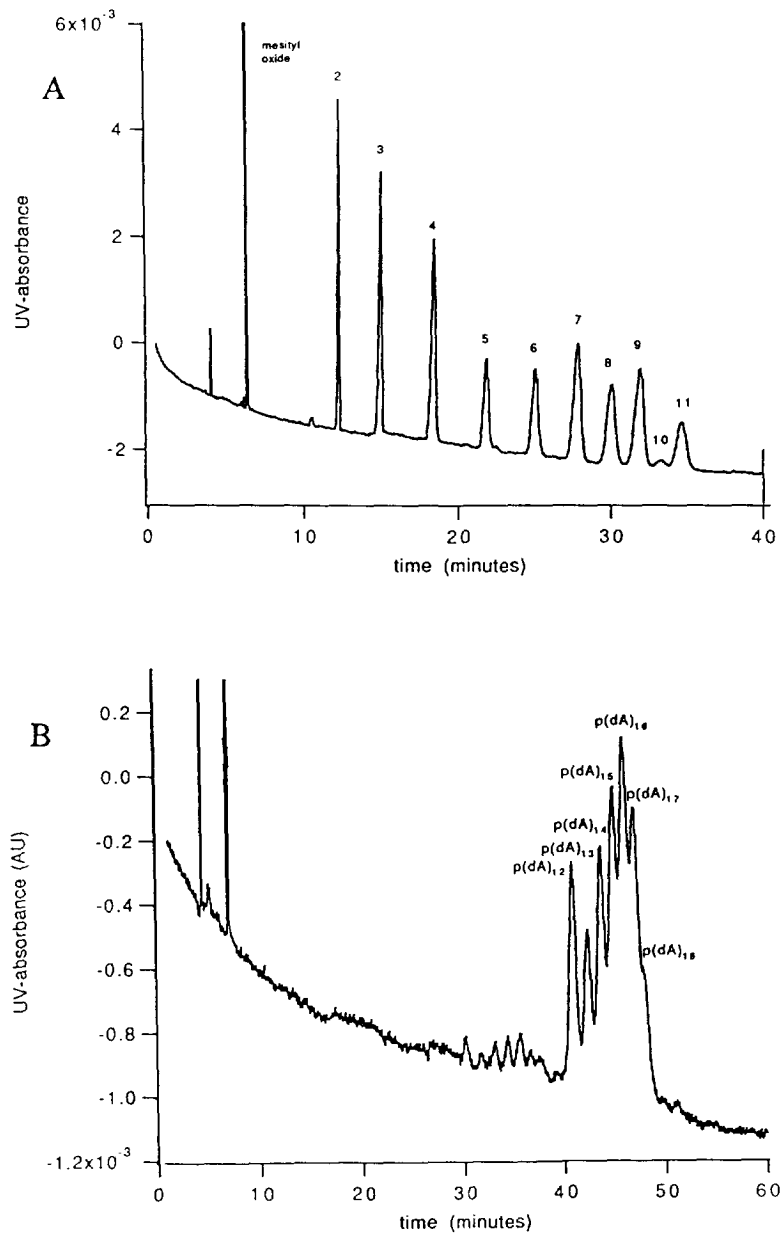

Fig. 1. Electropherograms of small desoxynucleotides in the free solution mode. (A) Separation of oligothymidylic acids $p(dT)_{2-11}$. Background electrolyte: 5 m*M* Tris, 5 m*M* NaH_2PO_4, 50 m*M* SDS, pH 7.0; Capillary, 55.7 cm × 50 μm I.D., effective length 44.7 cm; $V = 20$ kV, $I = 52$ μA; detection wavelength 260 nm; $T = 22$°C; injection, 15 mbar, 3 s. Numbers indicate the number of bases. (B) Separation of polydeoxyadenylic acids $p(dA)_{12-18}$. Experimental conditions as in (A).

Fig. 2 shows typical electropherograms obtained during the degradation. It can be seen that going from Fig. 2A to C the reaction rate decreases due to the lower exonuclease concentration. In Fig. 2A, where the nuclease concentration is 4 ng/μl, the reaction was nearly

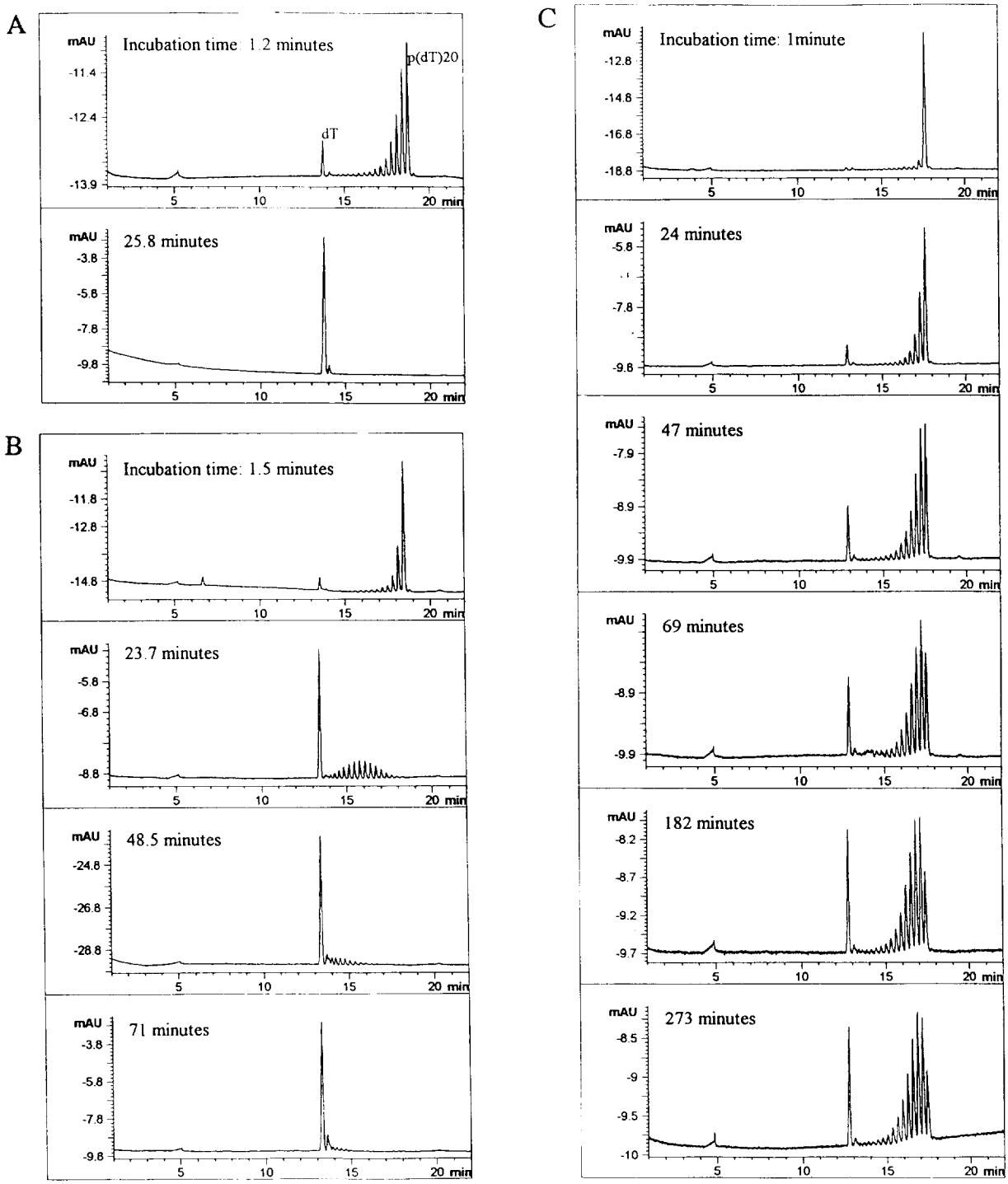

Fig. 2. Separation of $p(dT)_{20}$ and its degradants after incubation with snake venom nuclease. The electropherograms were obtained after injection at the indicated incubation times. Separation conditions: $V = -10$ kV, $I = 3$–4 μA; background electrolyte, 100 m*M* Tris, 100 m*M* boric acid, pH 8.5; capillary, 32 cm × 75 μm I.D., effective length, 24 cm; injection, −5 kV, 5 s. For incubation conditions, see text. Snake venom nuclease concentrations: (A) 4.0 ng/μl, (B) 0.95 ng/μl, (C) 0.22 ng/μl.

completed after 25.8 min incubation. At a concentration of approximately four times less snake venom nuclease, the reaction proceeds at a lower rate, as shown in Fig. 2B. In this case the reaction comes to completion after 71 min. In electropherograms A and B resulting from 1.2 and 1.5 min incubation time in A and B, respectively, baseline resolution between the $p(dT)_{20}$ peak and its degradants $p(dT)_{19}$, $p(dT)_{18}$, etc. can be observed. The increasing peak at 13.5 min is the single nucleotide p(dT). When p(dT)s with 2 to 5 bases are also present (at very low concentrations), these fragments coelute, as seen by the somewhat broader peak around 13.7 min.

At a nuclease concentration of 0.22 ng/μl the reaction rates are again slower. The reaction even stops after approximately two hours and no further change in the relative peak areas is observed, as seen in Fig. 2C.

Table 1 summarizes the relative standard deviations of the migration times and relative migration times, $t_r = t_{m,p(dT)n}/t_{m,p(dT)}$, for $p(dT)_{20}$ and some of its degradants. With RSD values between 0.3 and 0.5% an excellent reproducibility is observed. This is further improved by calculating relative migration times, using t_m of p(dT) as internal standard. However, it must be noted that this can only be achieved when the gel-filled capillary does not show bubble formation. In our experience, the gels could withstand between 30 and 50 injections without deteriorating in performance and/or in reproducibility. In our separation system, an indication for bubble formation at the injection side was a current below 3 μA, which could be confirmed under a microscope with 80 × magnification. The decreasing currents were accompanied by shifts in migration times and a drop in theoretical plates from ca. 200 000 to below 100 000. The possible problem with regard to identification of the oligonucleotide of interest and its degradants can be easily circumvented by using relative migration times.

Table 1
Average migration times and relative standard deviations (RSD), in migration times and relative migration times of four degradation products of $p(dT)_{20}$

	t_m (min)	RSD, t_m	RSD, t_r
dT	12.80	0.4	–
$p(dT)_{19}$	14.43	0.3	0.19
$p(dT)_{18}$	15.95	0.4	0.15
$p(dT)_{18}$	16.85	0.4	0.11
$p(dT)_{20}$	17.44	0.5	0.17

The extinction coefficient ϵ of an oligonucleotide increases strongly with its size. For example, the ϵ values for the monomer p(dT) and the 20-mer are $8.70 \cdot 10^3$ and $1.706 \cdot 10^5$ $l \cdot mol^{-1} \cdot cm^{-1}$, respectively. ϵ values can be calculated according to a method described by Puglisi and Tinoco [19]. An Excel program was used for this purpose in our lab [20]. The relative concentrations of $p(dT)_{20}$ and the degradants were calculated using computed extinction coefficients from the peak areas in the electropherograms as shown in Fig. 2. In Fig. 3, the relative concentrations of p(dT) and $p(dT)_{20}$ are plotted versus the incubation times at various nuclease concentrations.

In the reaction described above, which can be expressed as

$$p(dT)_{20} \xrightarrow{k_1} p(dT)_{19} \xrightarrow{k_2} p(dT)_{18} \xrightarrow{k_3} \ldots \rightarrow dT$$

the exponential decay of $p(dT)_{20}$ is given by

$$[p(dT)_{20}] = [p(dT)_{20}]_0 \cdot e^{-k_1 t}$$

where $[p(dT)_{20}]_0$ is the initial concentration and

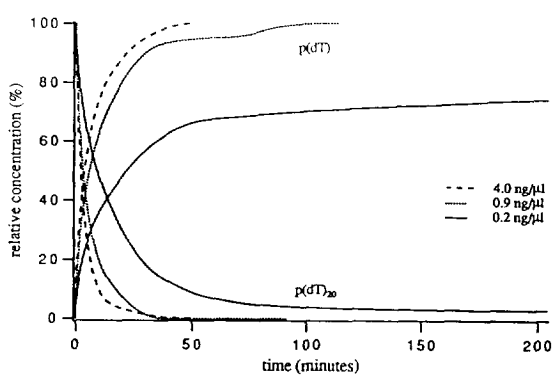

Fig. 3. Kinetic curves on incubation at three different snake venom nuclease concentrations. For incubation conditions, see text. Relative concentrations of $p(dT)_{20}$ and dT vs. time of incubation.

k_1 is the reaction constant of the first step. The subsequent increase and decrease in concentration of $p(dT)_{19}$ is given by

$$[p(dT)_{19}] = \frac{[p(dT)_{20}]k_1}{(k_2 - k_1)}(e^{-k_1 t} - e^{-k_2 t})$$

For smaller degradants similar equations can be derived.

The half-life time of each species $t_{1/2} = \ln 2/k_n$ can be derived from the computed rate constants.

Under these incubation conditions, $T = 24°C$, 40 m*M* Tris–HCl, pH 9.0, and the given concentrations of the reactants, the half-life times of $p(dT)_{20}$ were 2.1, 3.3 and 9.3 min, going from 0.4, to 0.95, to 0.22 ng/μl, respectively. It must be emphasized here that the half-life times have a strong dependence on the incubation conditions, such as the pH, type of media, the presence of Mg^{2+} ions, the temperature and the concentration of the reactants. For instance, addition of Mg^{2+} ions at physiological levels more than doubles the rate of degradation [20]. The degradation patterns found are fully in accordance with a 3'-end exonuclease activity and resemble results obtained from HPLC analysis of heterogeneous oligonucleotides as measured by Bacon et al. [6] and Vichier-Geurre et al. [21].

The high ionic strength of the sample solution resulting from buffer ions in the incubation vial largely reduces the amount of sample injected onto the gel-filled capillary. This is a well known phenomenon in CGE [22]. In Fig. 2, the drift and noise in the baseline are much more pronounced than in the case where an injection was made from a salt-free incubation vial (results not shown). In order to get rid of the buffer, and eventually other salts, a desalting step has to be used. This desalting step is strongly recommended for measurements in cell extracts where one has to deal with even higher salt concentrations, typically in the order of 100 m*M*. The effectiveness of the simple desalting step (see Experimental) is illustrated in Fig. 4A and B. In Fig. 4A, the reaction product p(dT) (from Fig. 2B, last electropherogram) was treated after a few hours according to the above described procedure and the sample was reinjected. A strong increase in peak area can be observed in Fig. 4B. [Before and during the desalting procedure, the small peaks in the upper electropherogram representing $p(dT)_{2-10}$, reacted all to p(dT).] The gain in peak area for the oligonucleotides between 15 and 20 bases in length is around a factor of 45 (see Fig. 5). Going to smaller fragment sizes the gain decreases because the smaller fragments are partly lost through the pores of the filter. However, even for the monomers a gain in peak area between 6 and 8 could be easily obtained.

Comparable information on degradation kinetics can be obtained with MALDI-MS. It has been shown to be a sensitive method with extremely high resolution for the analysis of both chemically modified and unmodified oligonucleotides [16,23]. MALDI-MS has the advantage that it also renders molecular masses of degradants. However, it is more complicated to perform the experiments with MALDI than in an automated CE fashion as described above, since mixing of the matrix and the sample (ca. 1.4 μl) from the incubation vial is essential before the sample is introduced into the vacuum system of the MALDI-time-of-flight mass spectrometer.

Some mass spectra of $p(dT)_{20}$ at different incubation times are depicted in Fig. 6. The difference between the peaks equals m/z 304, corresponding to the cleavage of p(dT) units from the p(dT)s. Also, peaks for doubly charged species, $(M - H)^{2-}$, and dimers, $(2M - H)^-$, are visible in the mass spectra.

3.2. Incubation of heterogeneous oligonucleotides with snake venom nuclease

With heterogeneous oligonucleotides the electropherograms show more irregular patterns, because both the electrophoretic mobility and thus the resolution and the peak height not only depend on the size of the oligonucleotide, but also on the base sequence [24,25]. This is illustrated in Fig. 7, where a 19-mer 5'-TTC TCG CTG GTG AGT TTC A-3' was mixed with snake venom nuclease in 40 m*M* Tris–HCl, 4

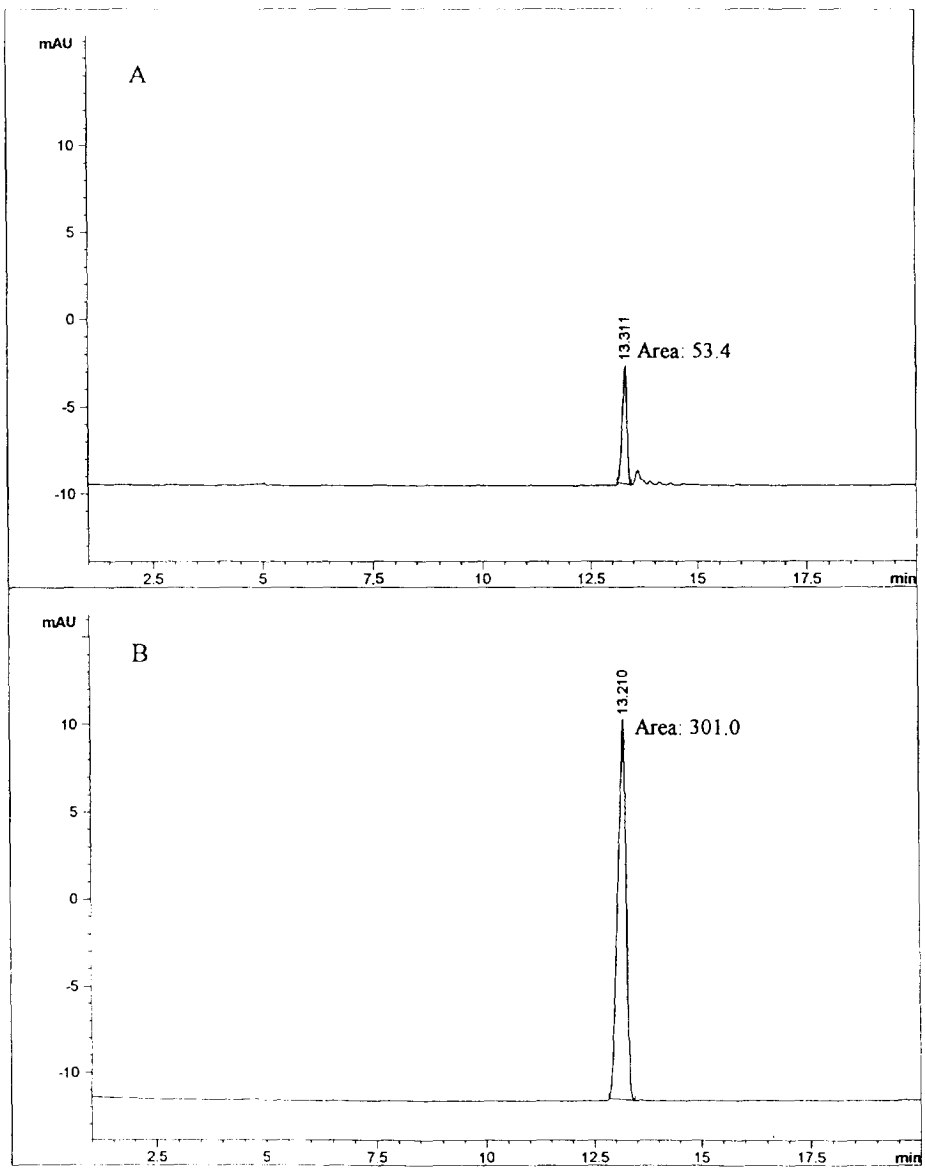

Fig. 4. The influence of sample ionic strength on amount of oligonucleotides injected. (A) Reaction product p(dT), dissolved in incubation buffer 40 m*M* Tris–HCl, 4 m*M* $MgCl_2$, pH 9.0. (B) Same sample after desalting.

m*M* $MgCl_2$, pH 9. The results from this 19-mer, a natural phosphodiester, are shown here and represent a 'reference' oligonucleotide for further experiments with chemically modified antisense oligonucleotides (see below); it consists of the same base sequence and length as the modified ones.

The small peak, migrating before the four monomers and marked with an asterisk, is the internal standard Orange G. The concentrations in the incubation vial were 0.005 μg/μl for the 19-mer and 4 ng/μl for the snake venom nuclease. In the fourth electropherogram (after 181 min incubation) one can observe the formation of a more or less stable intermediate. This intermediate is still present at much higher

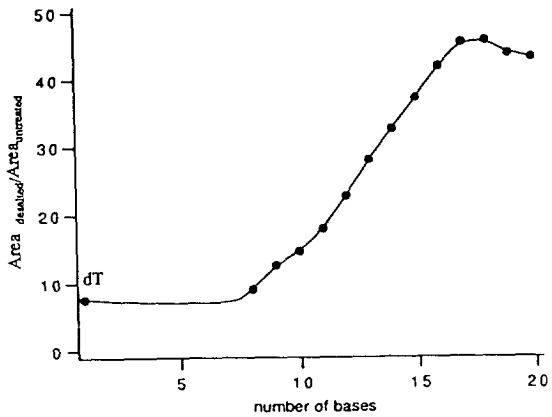

Fig. 5. The gain in peak area after the desalting step. The ratio of the peak areas of the oligothymidylic acids mixture before and after the desalting is plotted vs. the number of bases.

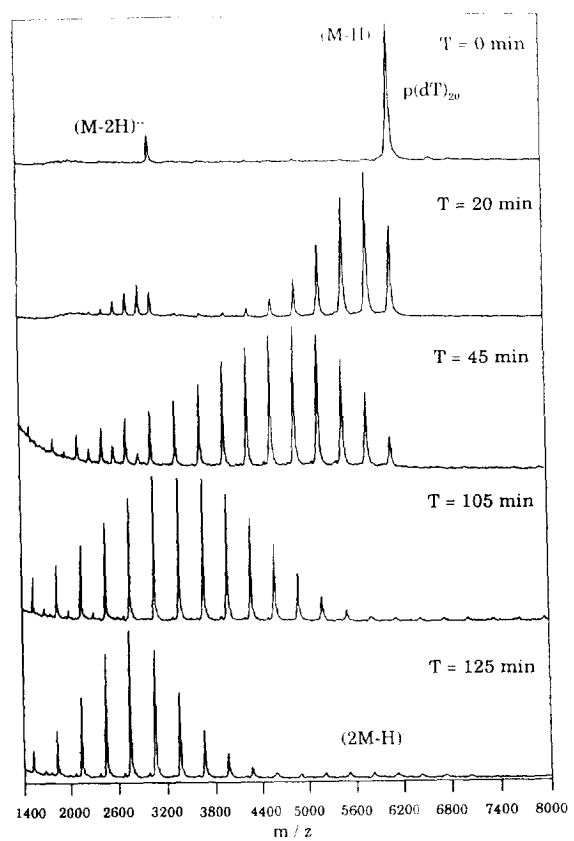

Fig. 6. MALDI-MS spectra of $p(dT)_{20}$ after incubation times with snake venom nuclease, using 2,6-dihydroxyacetophenon–diammonium hydrogencitrate as the matrix. For incubation conditions, see text.

concentrations than its parent compound and other degradation products. Probably, the presence of a certain base composition at the 3'-terminal residue shows a strong resistance to 3'-exonuclease attack resulting in slow degradation behavior. In the last electropherogram, resulting from 8.5 h incubation, the four remaining reaction products, p(dC), p(dT), p(dG) and p(dA) can be distinguished.

As already mentioned for the degradation of $p(dT)_{20}$ these cleavage reactions can also be followed in time with MALDI-MS. An example of a mass spectrum of a 25-mer 5'-TCA CAG CCT CCA CAC AGA GCC CAT C-3' after 80 min incubation with snake venom nuclease is shown in Fig. 8A. An advantage of MALDI-MS is that it also delivers molecular masses of the reaction products and thus gives more detailed information on reaction products than CE. The differences in m/z values between the main peaks correspond to the loss of one nucleotide subunit structure every time. The masses of pdC, pdT, pdA and pdG subunits are 289.2, 304.2, 313.2 and 329.2, respectively. The achieved mass resolution and molecular mass determination with MALDI-MS allow determination of the sequence of an oligonucleotide as can be seen in Fig. 8A. It must be noted here, that this mass spectrum was recorded without any further sample pretreatment. By desalting and by using an ammonium buffer, the quality and sensitivity can be easily improved [16]. With MALDI-MS, it should also be possible to clarify more complicated reaction schemes, e.g., reactions with nuclease S1 which acts both endo- and exonucleatically.

The physical behavior of digested oligonucleotides does not significantly differ in a time-of-flight mass spectrometer. However, the resolution and detection sensitivity decrease with increasing mass. Also, larger molecules need a higher laser intensity than small molecules. Therefore, quantitative information can be obtained only in a small mass range. Over broader mass ranges, peak intensities cannot be directly compared.

To compare MALDI-MS and CGE, the electropherogram of this oligonucleotide after 77

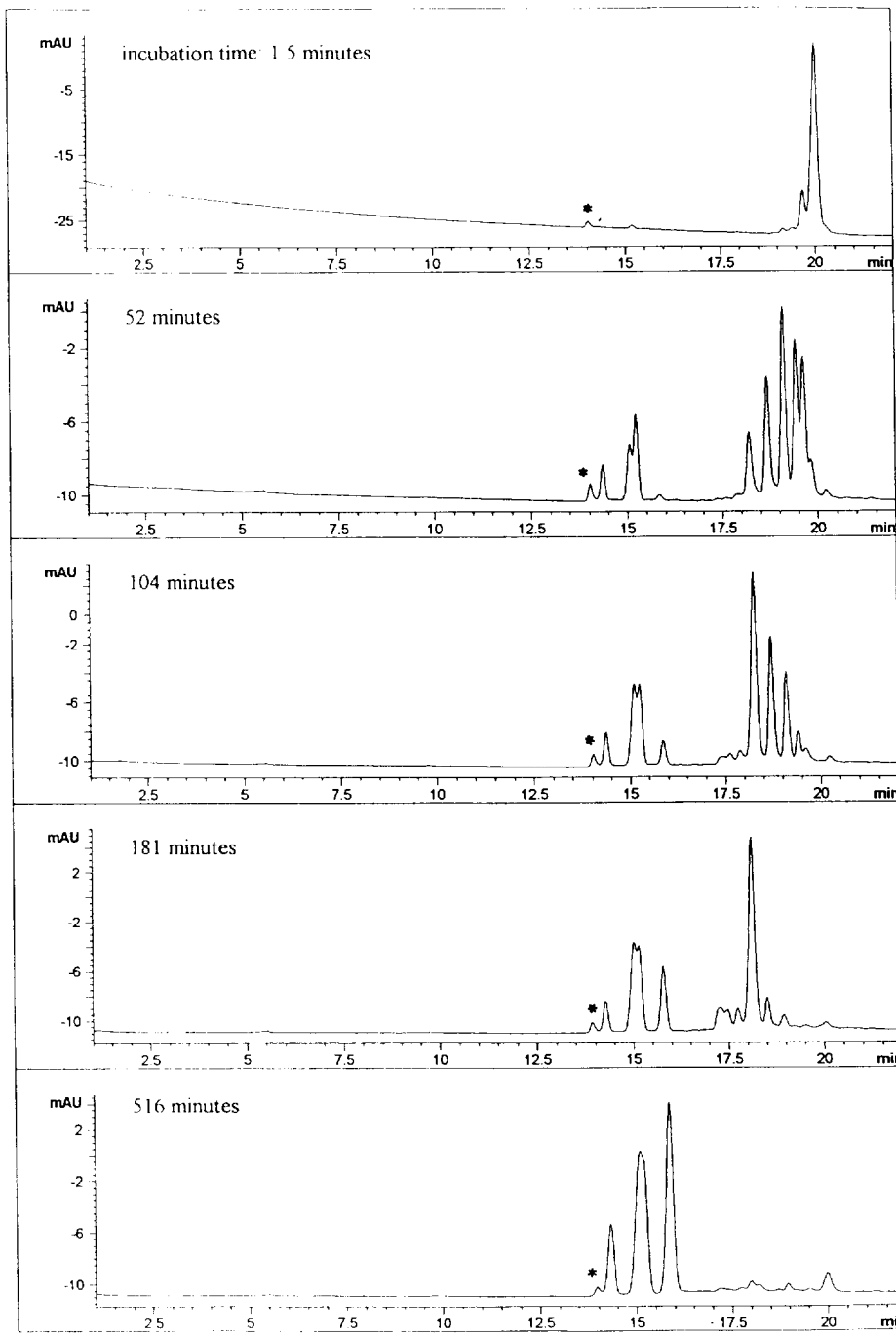

Fig. 7. Electropherograms of the degradation of a 19-mer 5′-TTC TCG CTG GTG AGT TTC A-3′ with snake venom nuclease at increasing incubation times. Experimental conditions as in Fig. 2. The Orange G peak is marked with an asterisk.

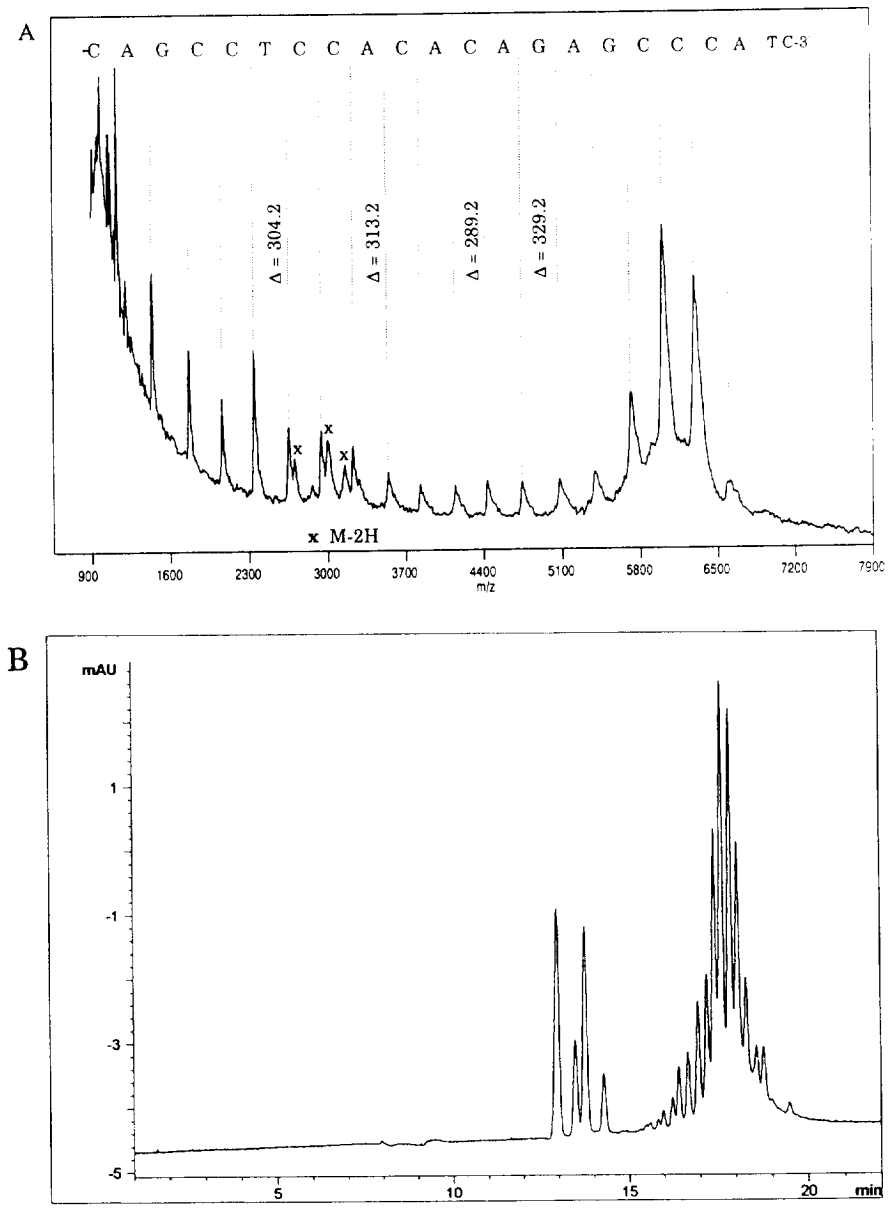

Fig. 8. (A) MALDI-MS spectrum of a 25-mer 5′-TCA CAG CCT CCA CAC AGA GCC CAT C-3′ after 80 min incubation with snake venom nuclease. The peaks marked with × are doubly charged fragments. (B) Electropherogram of the 25-mer after 77 min incubation. Experimental conditions as in Fig. 2.

min incubation is depicted in Fig. 8B. Whereas with MALDI-MS a better resolution between the peaks can be obtained, the electropherograms also deliver information about the relative concentrations of the formed monomers. Also, the quantitative interpretation of the data is more straightforward and more reliable than for MALDI-MS.

3.3. Incubation of a heterogeneous 25-mer with nuclease S1

The electropherograms look completely different when nuclease S1 instead of snake venom nuclease is used. Nuclease S1 hydrolyzes single-stranded nucleic acids endo- and exonnucleatically. In the electropherograms in Fig. 9, the reaction mixture consisted of 5.10^{-3} μg/μl 25-mer 5′-AGT GTC GGA GGT GTG TCT CGG GTA G-3′ (complementary to the string from Fig. 8) and 3.6 U/μl Nuclease S1 dissolved in water. The total reaction volume was not more than 11 μl. Only in the first two electrophero-

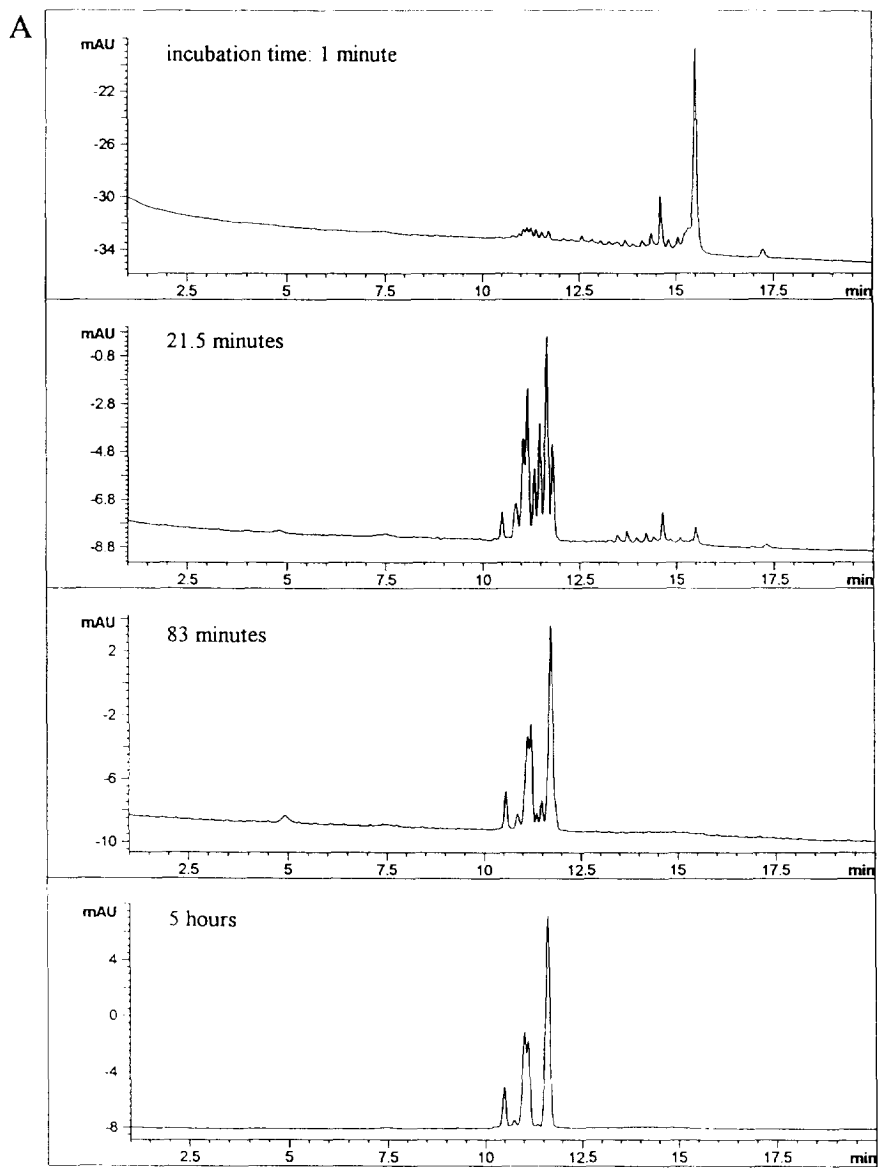

Fig. 9. Electropherograms of 0.005 μg/μl 25-mer 5′-AGT GTC GGA GGT GTG TCT CGG GTA G-3′ after incubation with Nuclease S1. Incubation in water, 3.63 μg/μl Nuclease S1, reaction volume 11 μl.

grams larger fragments can be observed. Already in an early stage of the degradation process the appearance of small fragments, such as the monomers and dimers, is visible. The last electropherogram shows a comparable peak distribution for the four monomers as in the case of incubation with snake venom nuclease.

3.4. Analysis of modified antisense oligonucleotides

As shown in the electropherograms above, oligonucleotides possessing the natural structure are hardly suitable for therapeutic development, because of quick degradation by various nu-

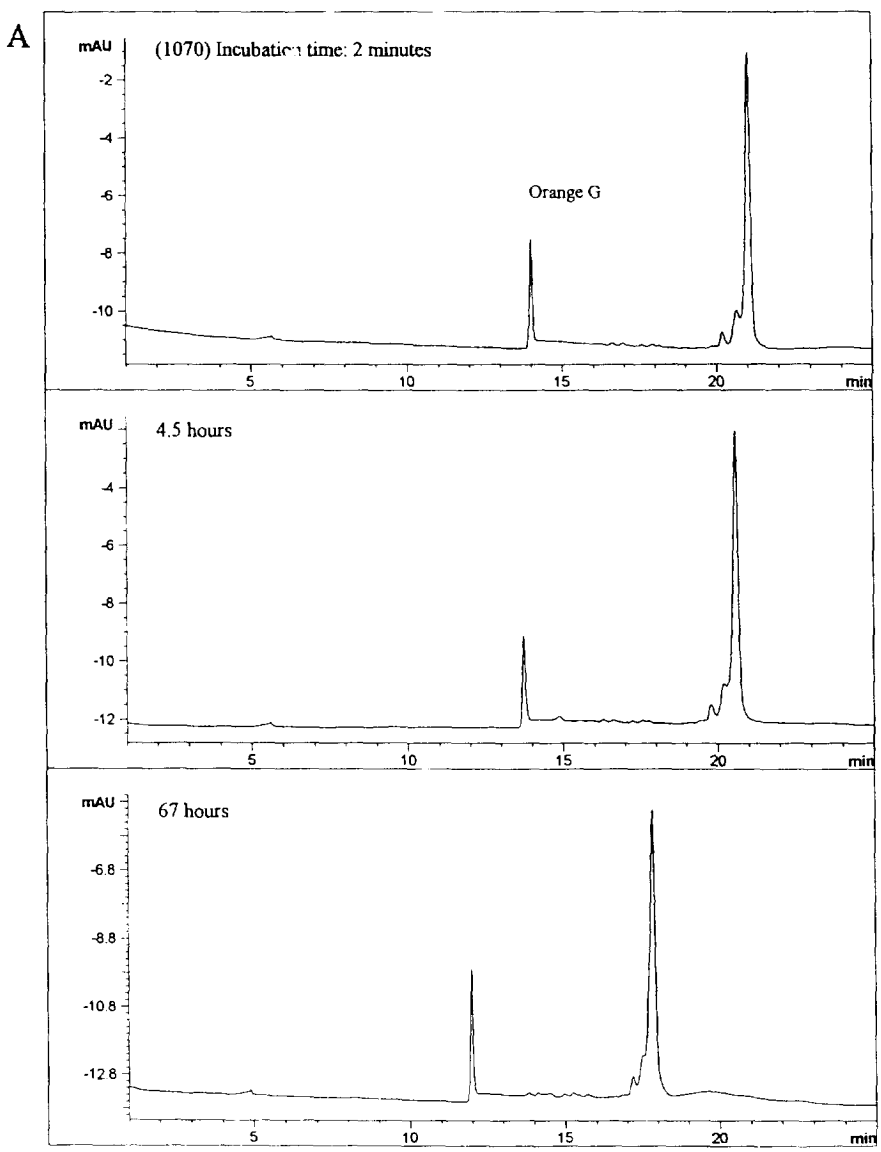

Fig. 10 (*continued on p. 194*).

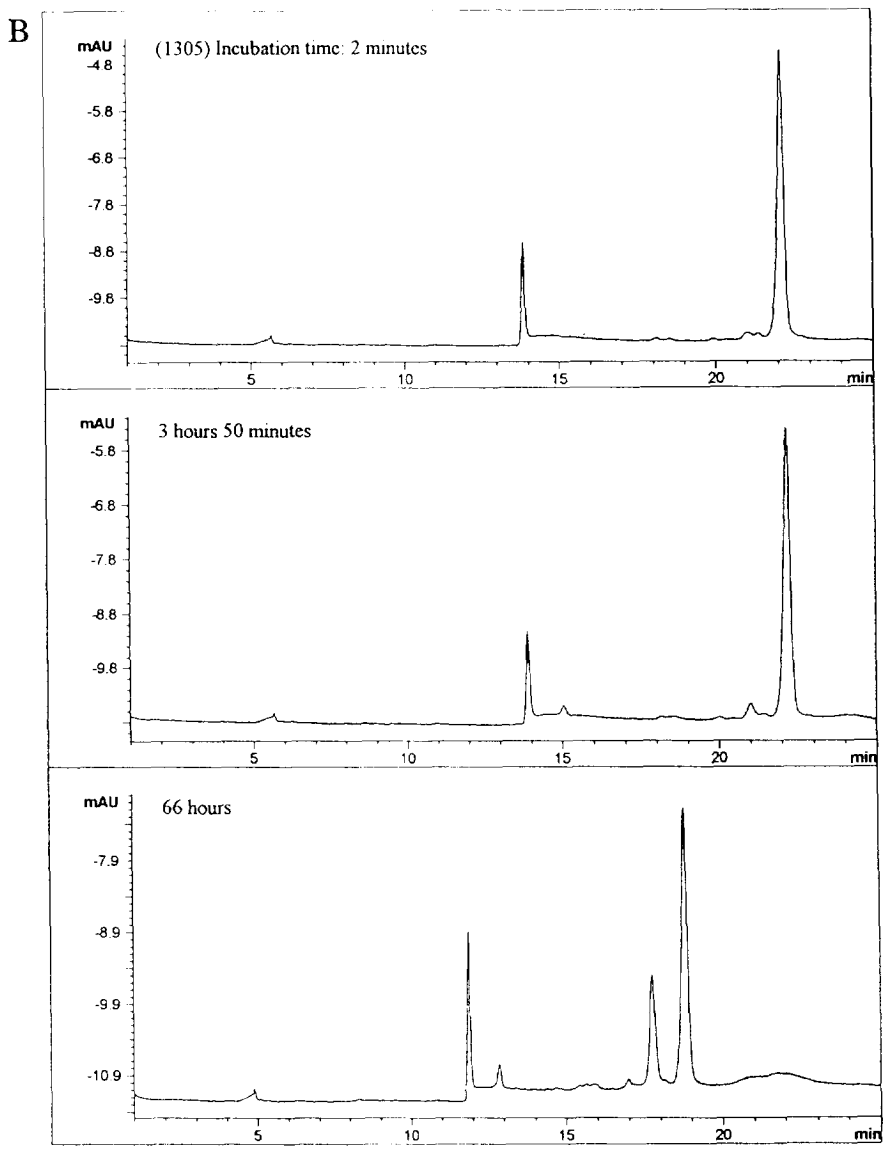

Fig. 10. (A,B) Electropherograms of two chemically modified antisense oligonucleotides after various incubation times. Incubation with snake venom nuclease. Experimental conditions as in Fig. 2.

cleases present in cell extracts. Numeral structural modifications have been proposed and synthesized to improve the resistance to nuclease degradation. Some examples of electropherograms of modified oligonucleotides, resulting from a comparative study, are given here in order to show the difference in stability from the natural phosphodiester. The sequence in these examples is the same as in Fig. 7. The difference consists of modifications in the base structures and the substitution of non-bridging oxygen by sulfur at various, defined positions in the backbone of the 19-mer. During the stability measurements of a series of modified oligonucleotides, which could be easily done in overnight runs, the unmodified phosphodiester was mea-

sured as well in order to verify reproducible half-life times with different nuclease and buffer solutions and capillaries, etc., and test the day-to-day reproducibility. The electropherograms are depicted in Fig. 10A and B. As shown, the oligonucleotide in Fig. 10A was much more stable than the natural phosphodiester. Whereas the half-life time of the unmodified oligonucleotide was only three minutes, the modified one given in Fig. 10A does not show any sign of degradation with snake venom nuclease after 67 h incubation. The antisense oligonucleotide in Fig. 10B develops one clearly distinguishable degradant. Also, the formation of one nucleotide migrating after the Orange G peak can be observed in time. The shift in migration time to lower values in both examples (from the second to the third electropherogram) is caused by the somewhat shorter capillary, which was installed in between those runs.

4. Conclusions

This study has shown that stability measurements of antisense oligonucleotides can be performed with CGE and MALDI-MS. Both methods have their own advantages and the type of information is complementary. Excellent reproducibility of migration times can be obtained with CGE, when care has been taken for a reproducible production of the gel-filled capillaries. In combination with the desalting method as described above, it is expected that the analysis of antisense oligonucleotides in cell extracts will be possible. This is currently under investigation in our laboratory.

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