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On-column electroextraction and separation of antisense oligonucleotides in human plasma by capillary gel electrophoresis

Anders K. Palm [a], György Marko-Varga [b,*]

[a] *Department of Chemistry, Indiana University, Bloomington, IN 47405, USA*
[b] *AstraZeneca R&D Lund, SE-22187 Lund, Sweden*

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Abstract

A novel approach is presented for the direct injection, and subsequent separation, of antisense phosphorothioate oligonucleotides in human plasma by capillary gel electrophoresis. The plasma, spiked with the antisense, was simply diluted 1:1 with acidified water and inserted into the sample holder in the capillary electrophoresis instrument. The separation capillary, filled with a dextran solution (replaceable polymer) and a short zone of acidified water at the injection side, was dipped into the plasma sample vial and voltage applied for simultaneous electrokinetic extraction and injection of antisense. The sample vial was then exchanged for the buffer vial, separation voltage applied, and size-sieving separation achieved. Separation time is less than 9 min and total time per analysis cycle 20 min, including rinsing of the capillary, filling with polymer, electroextraction/injection, and separation. This automated method can handle small sample volumes (4 μl) and has a detection limit of 0.5 μg ml^{-1} for a 16-mer phosphorothioate employing UV-detection. The capillary is stable for about 50 analyses.

Keywords: Oligonucleotides; Antisense DNA; Plasma; Sample extraction; Capillary gel electrophoresis

1. Introduction

Synthetic oligonucleotides (oligos) like phosphorothioates or similar modified antisense oligos has emerged as promising agents for drug target evaluation and therapeutics against disease [1,2]. Antisense oligos (typically 15–25 bases) most often consists of modified phosphodiester oligonucleotides where either the base-, ribose-, or backbone unit have been chemically modified to alter its resistance against nucleases and enhance the specificity and selectivity towards its target [3,4]. The antisense is directed against its complementary DNA or mRNA sequence to modulate or inhibit protein expression.

Following the development of antisense technology the need for fast analytical separations with high-resolution, high-detection sensitivity, and small sample volume consumption has increased. Polyacrylamide gel electrophoresis and high-performance

* Corresponding author. Tel.: +46-46-336887; fax: +46-46-337383.
E-mail address: gyorgy.marko-varga@astrazeneca.com (G. Marko-Varga).

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liquid chromatography (reversed-phase and anion-exchange mode) have long been applied for various antisense analyses [5–8]. Capillary zone electrophoresis of antisense was recently demonstrated [9,10]. Also, electrospray ionization mass spectrometry (ESI-MS) [11], capillary gel electrophoresis on-line to ESI-MS [12] and matrix-assisted laser desorption/ionization mass spectrometry [13,14] are frequently used for such analyses and adds other analytical information such as, e.g. molecular weight.

To date, capillary gel electrophoresis (CGE) seems the most prefered separation technique and has the highest resolving power [6–8,11–21]. Single nucleotide baseline separation of smaller and larger antisense, simultaneously, is here achieved. The sieving matrix in CGE most often consists of a low viscosity polymer which is easily pushed out of the capillary after a run and replaced by a fresh matrix for a subsequent analysis (typically a pressure of a few bars is enough to displace such a matrix on a minutes time scale, which is standard on commercial instruments) [22,23]. Hereby the more difficult in-situ gel polymerization techniques can be avoided since they are more prone to problems regarding gel preparation, memory effects, being more sensitive to clogging, as well as having a limited lifetime [24].

Several sample clean-up approaches (occasionally combined with preconcentration) have been on-line interfaced to various modes of capillary electrophoresis (CE) and demonstrated for the extraction and separation of biomolecules from biological fluids, for example, microdialysis [25], electrodialysis [26], and liquid–liquid electroextraction [27,28], Also, direct CE injections of pharmaceuticals from plasma sample without pretreatment has been demonstrated [29] and more recently injection of proteins directly from urine followed by CGE separation was shown [30]. However, direct injection of oligos from plasma sample into a fused-silica capillary followed by CGE separation have, to the authors' knowledge, not been demonstrated. While CGE with low viscosity replaceable polymers can tolerate less clean samples than capillaries containing immobilized gels it is here common practice to extract, purify and concentrate antisense from biological fluids off-line before injection into the capillary. Extraction of antisense from tissue, plasma, or serum is mostly devoted to liquid–liquid extractions, e.g. by phenol/chloroform, and/or solid-phase extraction techniques like ion-exchange and reversed-phase [8,9,11,13,15–21]. These techniques often affords adequate yield and reproducibility. They are, however, laborious and time consuming, and sample volumes on the order of 50–100 μl or larger volumes are often necessary. Other technique use adsorption/affinity beads (anionic and hydrophobic) that selectively captures any phosphorothioate oligo in a sample. The oligos are subsequently washed to remove contaminating material, released from the beads, and injected and analyzed by CGE or mass spectrometry [31]. By capillary affinity gel electrophoresis it was recently demonstrated the on-column capturing and analysis of phosphodiester oligos [32,33]. The complementary oligo sequence to the one of interest in sample was immobilized to the size-sieving polymer as to selectively capture the sample oligo during injection. The captured oligo was later released by increased temperature or urea concentration followed by separation. This technique partially discriminates against oligo sequence and composition and is therefore suitable also for nucleotide mismatch analysis. A high detection sensitivity is affordable due to the on-column concentration step. However, no analysis of oligos from biological fluids was shown. Recently, it was demonstrated an integrated on-chip approach of solid-phase extraction and purification of DNA from a biological matrix followed by polymer size-sieving electrophoresis, i.e. all steps were performed on a single microfabricated device [34].

Oligos are mostly detected by their intrinsic UV absorbance (254–260 nm) and low femtomole levels (10–100 fmol range) can be reached when analyzed by CGE, especially when field-amplified injection is used [35]. Similar to CE in general, however, the concentration has to be fairly high, in the nanomolar range, corresponding to low microgram—high nanogram of oligo per milliliter sample. To enhance the detection sensitivity of antisense in CGE laser-induced fluorescence detection [13] and isotachophoresis in partially polymer-filled capillaries [36,37] have been reported.

In this report we present a method for the simultaneous electroextraction and injection of phosphorothioate oligonucleotides directly from human plasma into a fused-silica capillary for subsequent analysis by

CGE. The sample treatment is simple and the technique is suitably for oligonucleotide extraction from small volumes of biological fluids. The method has demonstrated low detection limits, high reproducibility, short analysis time, with the results being quantitative.

2. Experimental

2.1. Chemicals

Coated fused-silica columns with i.d. 100 μm (o.d. 360 μm) (eCAP neutral capillary, DNA) were purchased from Beckman Coulter (Fullerton, CA, USA). Phosphorothioate oligonucleotides 3′-GACTGACTGACTGACT-5′ and 3′-GACTGACTGACTGACTGACT-5′, 16 and 20 bases, were from Amersham Biosciences (Uppsala, Sweden). Dextran (MW: 464,000), boric acid, tris(hydroxymethyl)aminomethan (Tris), sodium dodecyl sulphate (SDS), urea and formic acid (free acid, minimum 95%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared using Milli-Q water (Bedford, MA, USA).

2.2. Instrumentation

All analyses were performed on a Beckman P/ACE 5510 capillary electrophoresis instrument (Fullerton, CA, USA). No external cooling of sample carousel was used. The cartridge holding the capillary had an 100 μm × 800 μm aperture. Detection, UV absorbance at 254 nm. Cooling temperature of capillary was set at 30 °C, 1 s rise time, 5 Hz data rate.

2.3. Buffer, polymer/acid capillary filling, and capillary rinse procedure

The buffer consisted of 210 mM boric acid, 110 mM Tris, pH 8.0, containing 7 M urea; filtered through a 0.45 μm PVDF syringe filter. One milliliter of a 16% (w/v) dextran solution was prepared by dissolving the appropriate amount of dextran in buffer (no attention was paid to the small volume increment). After dissolving the polymer for 1 h by slow shaking the solution was ultrasonicated for 5 min to remove air bubbles. Then 100 μl was transferred into an eppendorf tube and placed in the CE sample holder. The polymer was pressurized (20 psi) through the capillary for 4 min, the capillary having a total length of 27 cm (20 cm to detector). The dextran inlet vial was then exchanged for a vial containing 0.01% (v/v) formic acid in water and pressure (20 psi) was reapplied for 0.2 min. The capillary was then ready for electroextraction/injection of sample and separation. After both the polymer filling step and plasma sample injection step the capillary was dipped into water. Between each run the capillary was rinsed (20 psi) for 2-, 0.5-, 0.5-, and 1-min each with 0.05% SDS, 10% ethanol, 0.1% formic acid and water, respectively. The polymer solution was stable for at least 4 weeks when stored at 6 °C. Buffer vials (4.5 ml each) were changed every 10th run.

2.4. Plasma sample

Freshly prepared EDTA human plasma from healthy volunteers were stored at −18 °C in small aliquots over the experimental period (about 5 months). A new aliquot was taken on a daily bases and centrifuged for 2 min at 5000 rpm (6 °C) in an eppendorf style centrifuge before use. During the course of the day this plasma was stored on ice.

2.5. Sample treatment

A stock solution of 2 mg ml^{-1} in water was made of each phosphorothioate, PS-16 (16 bases) and PS-20 (20 bases). The oligos were mixed and further dilutions made in water. Five microliters of plasma was spiked with 0.25 μl of the mixed oligos at various concentrations, see below (i.e. 5% dilution of plasma only) and stored on ice for 5 min (“simulating” antisense distribution in plasma). To this was added 5 μl of 0.02% formic acid.

2.6. Electroextraction/injection and separation

The sample was inserted into the CE instrument. Before extraction/injection started there was a delay time of about 10 min due to rinsing, polymer-, and formic acid filling of the capillary. Extraction/injection was performed at 2.7 kV (100 V cm^{-1}) for 6 min. Separation voltage was 13.5 kV (500 V cm^{-1}).

3. Results and discussion

3.1. Preliminary observations

Injection of antisense oligos from a biological fluid like plasma directly into a coated fused-silica capillary, most of which is filled with a polymer solution, puts high demands on the stability of the polymer matrix and capillary surface coating for efficient and reproducible separations. During injection also plasma proteins and other plasma constituents will migrate electrokinetically and by diffusion into the column where they can potentially interfere with the qualitative and quantitative analysis, for example, by interacting with the oligos during separation whereby their resolution and sensitivity might decrease as well as interacting reversible/irreversible with the polymer and capillary surface and thereby alter their physicochemical properties. The importance of an efficient capillary surface rinsing scheme under such circumstances are described in [29,30]. It is also a known phenomenon in CE that a high ionic strength in the sample, as in biological fluids, relative the ionic strength of the background electrolyte used during separation gives a low sample injection yield and low efficiency separation [35]. These drawbacks might obviously become less the more the plasma is diluted but potentially at the expense of detectability, if not, for example, an on-line sample capturing could be used [32,33].

Initially, the whole/raw plasma was spiked with the phosphorothioates PS-16 and PS-20 (50 μg ml^{-1} each) and electrokinetically injected directly into the partial polymer, partial acidified water filled capillary. The efficiency of the separation was, however, too low and the lifetime of the capillary was limited to one or a few runs only after which it had to be discarded. Therefore, a dilution series of the plasma was made keeping the concentration of the PS oligos constant (50 μg ml^{-1}). The dilution was made in acidified water (0.01 % formic acid, final concentration). Results indicated a 1:1 dilution to be most efficient. Further dilution of both plasma and sample (1:4, 25 μg ml^{-1} each of respective oligo) gave a similar peak area and height for the PS-16 oligo as seen in the 1:1 dilution but a broader or sometimes splitted peak was observed for the PS-20 oligo. Peak splitting of DNA in capillary gel electrophoresis has earlier been shown to occur with a sample matrix containing sodium chloride [38].

3.2. Demonstration of antisense separation in an optimized matrix after electroextraction/injection

The electropherogram in Fig. 1 demonstrates a CGE analysis of the PS-16 and PS-20 oligos after electroextraction/injection from a plasma sample. Some

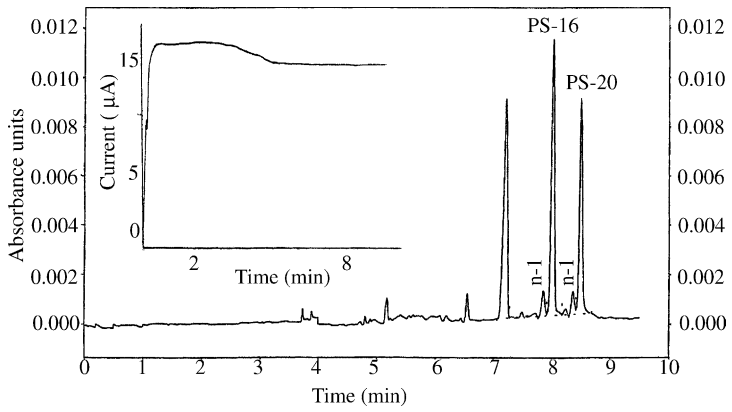

Fig. 1. Electropherogram demonstrating the separation of phosphorothioate (antisense) oligonucleotides by capillary gel electrophoresis. The antisense were electroextracted/injected from human plasma on-column. Extraction/injection proceeded for 6 min at 100 V cm^{-1}. Separation was at 500 V cm^{-1}. Sample: 50 μg ml^{-1} each of PS-16 and PS-20 spiked into human plasma and diluted 1:1 in acidified water. Capillary: coated, i.d. 100 μm, 20 cm to detector, filled with dextran polymer and a short zone containing 0.01% formic acid. Buffer: Tris–borate (210/110 mM), pH 8, 7 M urea. Temperature: 30 °C. Detection: 254 nm.

small peaks as well as an irregular baseline was seen between 4 and 7 min while a larger peak appeared at 7.2 min. These peaks could be plasma constituents and/or system peaks originating from the injection procedure [39]. The migration time and peak area of the larger and smaller peaks were highly reproducible and could potentially be used as internal standards. The insert in Fig. 1 illustrates the current profile during the CGE separation. At the beginning of the analysis the current increased according to the voltage ramp (0.25 min voltage ramp), between 0.5 and 3 min it remained constant (16.5 μA), and between 3 and 5 min it declined (from 16.5 to 14.5 μA). After 5 min it stayed the same during the rest of the run. This current behavior was due to excess ions from the plasma sample leaving the capillary. The small peaks appearing in close proximity of PS-16 and PS-20, at 7.8 and 8.4 min, respectively, are most probably failure sequences (n-1, etc.) from the synthesis of respective oligo. This is often seen in high-resolution separations of synthetic oligos where either the full-length oligo has not been fully purified after synthesis [6] or the oligo is degraded with time, for instances by nucleases. The ratios of peak area of respective full-length oligo over its deletion sequences remained constant during the entire experimental period. In a blank run (sample in buffer, no plasma) when overloading the column with PS-16 and PS-20, also n-2 to n-4 deletion sequences appeared while not base-line resolved (not shown). The yield of the full-length PS-16 and PS 20, over its respective deletion sequences, were appreciated to about 85% each based on their corrected peak area ratios. Furthermore, the peak height and area was less for PS-20 than PS-16; about 20% less peak area. The concentration of each oligo was 50 μg ml^{-1} × 0.85 (50 μg ml^{-1} as measured by spectrophotometry at 254 nm), and since they have the same base composition and nucleotide sequence a similar absorbance detection area response should follow according to Beer's law (molar ratio for PS-16 over PS-20 is 1:0.8 while absorption coefficient ratio is 0.8:1, any secondary structures are not expected according to the manufacturer). While it earlier has been recognized there is a stacking process and high selectivity for DNA crossing a free electrolyte–polymer solution interface in CGE [40] this bias was not seen here in a blank run where the oligos showed a similar peak area and height response. One possible reason for this injection bias with the plasma sample could be a higher affinity for the PS-20 oligo than the PS-16 oligo towards plasma constituents. Less PS-20 would therefore be free (non-bound) and available for injection. It is known that phosphorothioates are "sticky" and bind nonspecifically to proteins [21,41]. On the other hand, according to the graphs in Figs. 2 and 3 where the extraction/injection time (Fig. 2) and sample concentration (Fig. 3) are plotted against respective PS-16 and PS-20 oligo peak area and height, the graphs converge at lower injection times and concentrations, respectively. If this is correct the peak area and height discrepancy observed between the antisense oligos is an effect due to the injection process whose origin is not known to the authors. In Fig. 2 linear graphs were obtained in the injection interval tested; 90–450 s. For the PS-20 oligo, however, an injection time of 360 s was found maximal since beyond that the separation

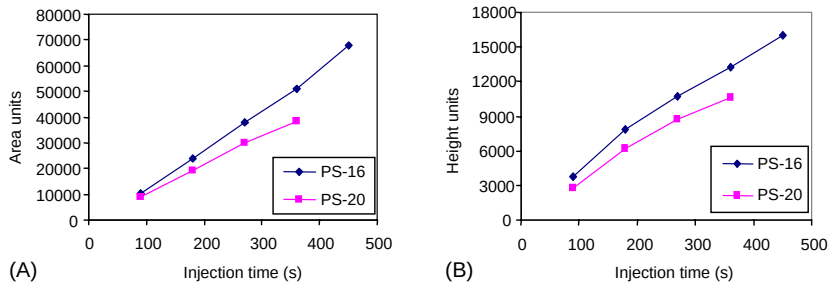

Fig. 2. Graphs of peak area (A) and height (B) vs. electroextraction/injection time of phosphorothioates from human plasma, as analyzed by capillary gel electrophoresis. Similar conditions as in Fig. 1. The following correlation coefficients were obtained from linear regression analysis: (A) PS-16, $r^2 = 0.9978$; PS-20, $r^2 = 0.9968$; (B) PS-16, $r^2 = 0.9916$; PS-20, $r^2 = 0.9824$. An injection time more than 360 s was not feasible with the PS-20 oligo due to anomalous behavior while PS-16 continued to show a linear increase up to 450 s.

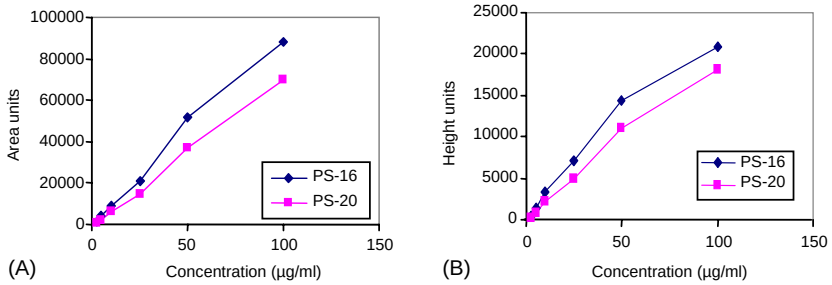

Fig. 3. Graphs of peak area (A) and height (B) vs. concentration (2.5–100 $\mu g\,ml^{-1}$) of phosphorothioates in human plasma as analyzed by capillary gel electrophoresis. Similar conditions as in Fig. 1. The following correlation coefficients were obtained from linear regression analysis: (A) PS-16, $r^2 = 0.9955$; PS-20, $r^2 = 0.9987$; (B) PS-16, $r^2 = 0.9647$; PS-20, $r^2 = 0.9885$. The lowest concentration detectable of PS-16 was 0.5 $\mu g\,ml^{-1}$ (S/N = 5).

efficiency decreased rapidly, the peak became broad, asymmetric, and sometimes splitted. PS-16, on the other hand, continued to show a linear response up to 450 s (the longest injection time tested). An injection time of 360 s (6 min) was therefore chosen throughout this evaluation. This is about two orders of magnitude longer injection time than "normally" applied in CE (at this electrical field strength). In Fig. 3 linear graphs were obtained over the concentration interval tested; 2.5–100 $\mu g\,ml^{-1}$ (datapoints from single runs). The lowest concentration detectable of PS-16 was 0.5 $\mu g\,ml^{-1}$ (S/N = 5).

Table 1 shows the compiled data of migration time, peak area and height reproducibilities for PS-16 and PS-20 for five consecutive runs on day 1 and day 5, respectively (in between day 1 and day 5 the capillary was used for ca. 40 analyses). Efficiencies varied between 5.5×10^5 and 7.5×10^5 plates m^{-1} and asymmetries between 0.8 and 1.1. The capillary typically lasted for about 50 analyses whereafter it had to be discarded. This was manifested as a general, quite abrupt, decrease in separation performance, especially the peak height of PS-20 which started to show large variations (see day 5 in Table 1). The RSD value of migration times could be improved to below 1.5% by normalization with respect to the migration time for the large peak appearing just before the antisense oligos (see Fig. 1).

Table 1
Migration time, peak area and height reproducibility[a]

	Migration time (% R.S.D.)	Peak area (% R.S.D.)	Peak height (% R.S.D.)
Run-to-run ($n = 5$), day 1			
PS-16	3.4	5.8	6.1
PS-20	3.6	4.5	6.5
Run-to-run ($n = 5$), day 5			
PS-16	3.9	6.3	5.7
PS-20	4.1	6.8	10.1

PS-16: phosphorothioate containing 16 bases; PS-20: phosphorothioate containing 20 bases.

[a] The analytical conditions were the same as shown in Fig. 1.

While in the above demonstration a 10 µl sample volume was used also a 4 µl sample volume (2 µl plasma sample + 2 µl acidified water) showed a similar performance.

3.3. Effects of the acidic zone in the capillary

The acidic zone (0.01% formic acid in water, pH ~3.2) injected into the capillary after the polymer solution, and residing between the polymer and plasma sample during extraction/injection, was of major importance for the longevity of the capillary, the high resolution obtained between the antisense oligos, and the extraction yield (while the yield was independent of whether acidified water or buffer was used, Fig. 4). pH gradients in CE have earlier been reported as to enhance the selectivity between analytes during the course of a run or to concentrate sample species on-line [42–44]. The length of the acidic zone in the capillary was studied. It was found that when the pressure (20 psi) injection time of the acid was varied between 0.2 and 1.2 min a maximum extraction yield (as determined by peak area) of the PS-16 and PS-20 oligos were obtained at 0.6–1.0 min. The yield here was

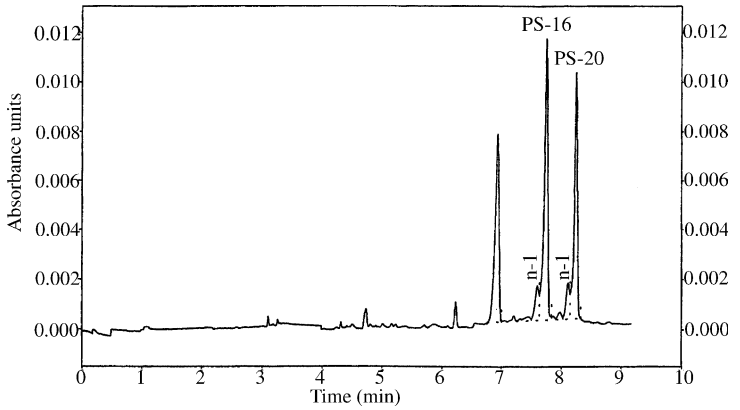

Fig. 4. Electropherogram demonstrating the separation of phosphorothioate (antisense) oligonucleotides by capillary gel electrophoresis. The antisense were electroextracted/injected from human plasma on-column. Similar conditions as in Fig. 1 were used except that the acidic zone (injected after the polymer, and residing between the polymer and the injected sample) was exchanged for Tris–borate–urea buffer (buffer zone). A similar yield was obtained as for the acidic zone but the selectivity decreased. This is illustrated by the n-1 failure sequences that extensively overlap with respective full-length oligo.

about two times higher than at 0.2 min. A similar separation efficiency for the oligos were obtained at all injection times but their migration times increased with an increased acidic zone length. However, run-to-run reproducibility of migration times and peak areas was poor for injection times larger than 0.2 min (as occasionally displayed by an anomalous current profile followed by broad and late eluting peaks). Maximum reproducibility was achieved at 0.2 min (see Table 1). Also, the concentration of formic acid in the acidic zone was evaluated. Higher and lower concentrations than 0.01% were found less efficient with regard to yield and efficiency of separation. Interestingly, the conductivity of the 0.01% formic acid water solution was similar to the Tris–boric acid–urea buffer in the polymer solution (as measured by Eq. (88) in [45]). The length of the acidic zone in the capillary at 0.2 min injection time corresponded to between 1.5 and 2.5 cm. These figures were obtained by pressurizing at 20 psi either polymer solution or formic acid from the inlet vial through the polymer filled column. A colored agent (Vitamin B_{12}) was dissolved in polymer and acid, respectively, and the time for it to appear at the detector was measured at the inflection point on the sigmoidal curve (similar to frontal analysis, see [46]). The length of the zone was then simply obtained by dividing the injection time (0.2 min) with the corresponding time at respective inflection point (1.6 min for the acid and 2.6 min for the polymer, respectively) times the capillary length to detector (20 cm). An acidic zone length closer to 1.5 cm than 2.5 cm seems reasonable due to the short injection time of the acid, i.e. the viscous resistance due to the polymer is dominating.

The injected acidic zone was exchanged for a zone of Tris–borate–urea buffer (residing between the polymer and plasma sample). An extraction yield of the antisense oligos similar as for the acid was obtained while their selectivity decreased. This is illustrated in Fig. 4 where the n-1 failure sequences extensively overlapp with respective full-length oligo. If no liquid zone at all was injected into the capillary (i.e. the whole capillary was filled with polymer) the extraction yield decreased extensively (about 75% less) however, retaining a similar resolution as above. Using any of these two latter approaches the performance of the separation started to decline after only a few runs whereby the column had to be discarded since no rinsing scheme could restore its performance.

3.4. Effects of acidified plasma

The plasma was diluted 1:1 in acidified water (0.01% (v/v) formic acid, final concentration). For reasons similar to above this was made in an attempt to further increase the robustness of the separation

system and capillary longevity. Since the majority of proteins, and especially albumin, in plasma are acidic (isoelectric points between 4 and 7), while less acidic than phosphorothioates [41], a decrease in pH where thought to selectively reduce or eliminate their electromigration into the capillary. However, while no detectable pH decrease in the plasma sample was observed (as judged by pH paper indicator), it was the authors experience that by acidifying the plasma a better separation reproducibility was achieved, and maybe to a lesser magnitude extending the lifetime of the column. A dilution of the plasma in water solely seemed here less favorable. Furthermore, dilution in higher formic acid concentrations (up to 0.1%, v/v) of the plasma was evaluated. This, however, resulted in flocculation/aggregation of plasma constituents followed by a concomitant decrease in the PS-16 and PS-20 yield during injection. Also, acetic acid was evaluated. Similar results were displayed as with formic acid. Other means, such as the addition of acetonitrile (10–50 %) in the plasma to selectively precipitate proteins, etc., was not successful [28]. It has earlier been reported the occlusion of phosphorothioates with proteins in plasma during denaturing extraction [8].

3.5. Capillary rinse scheme

The capillary rinse scheme between each run, consisting of 0.05% SDS, 10% ethanol, 0.1% formic acid, and water, was of major importance for the reproducible high-performance separations and capillary lifetime. Higher concentrations of SDS and ethanol seemed to negatively affect the performance of the capillary, however, their optimal concentrations still remains an issue. While not evaluated herein, other capillary coatings, rinse solutions and rinse times, might eventually aid in prolonging the lifetime of the column.

4. Conclusions

We have demonstrated an automated, rapid, and simple method using on-column electroextraction/injection and separation of antisense oligonucleotides from human plasma by capillary gel electrophoresis. No other pretreatment of the plasma sample other than a 1:1 dilution in acidified water was necessary. A low detection limit, high migration time-, peak area-, and peak height reproducibility was achieved allowing quantitative and sensitive analyses. Sample volumes as small as 4–10 μl could be handled as typical used in capillary electrophoresis.

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