ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1101–1105

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Capillary electrophoresis of oligonucleotides: micellar system or entangled polymers[1]

K. Khan, A. Van Schepdael*, J. Hoogmartens

Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, Van Evenstraat 4, B-3000 Leuven, Belgium

Received for review 6 October 1995; revised manuscript received 27 November 1995

Abstract

The capillary electrophoretic separation of oligonucleotides (12–24 bases in length) using a micellar system or an entangled polymer solution was compared. Micellar electrokinetic capillary chromatography was performed using a fused-silica capillary whereas a J&W DB-17-coated capillary was used for capillary electrophoresis in entangled polymer solution. In both cases, a number of parameters were evaluated to improve separation. For example, the influence of concentration of copper(II) ions and sodium dodecyl sulfate, variation in applied voltage and temperature were investigated in the micellar system. The influence of variation in pH was also studied. The two methods were also compared with respect to their efficiencies. The results of quantitation showed better within-day and day-to-day repeatability for the entangled polymer solution. Both methods gave a comparable limit of detection of about 30 pg at a signal-to-noise ratio of 3.

Keywords: Capillary electrophoresis; Entangled polymer solution; Micellar electrokinetic capillary chromatography; Oligonucleotides

1. Introduction

Oligonucleotides (generally 12–30 bases in length) are currently being investigated as potential drugs for the treatment of cancer and viral infections [1]. Synthetic oligonucleotides have previously been analysed and purified by reversed-phase [2] and ion-exchange liquid chromatography (LC) [3] using gradient elution. These methods require large amounts of sample and are well suited for preparative purposes. Traditionally, polyacrylamide slab gel electrophoresis has been extensively used in the past [4]. This technique has the disadvantage of post-electrophoresis visualization of DNA by radio-labeling or staining, which is only semi-quantitative. Capillary electrophoresis (CE) allows the use of a higher field strength and therefore it is more efficient. The advantages of CE are speed, on-line sample detection, easy quantitation and automation. Different modes of CE can be evaluated for oligonucleotide separation. However, capillary zone electrophoresis (CZE) cannot be applied to resolve oligonucleotides which are 12–24 bases in

* Corresponding author.

[1] Presented at the Fifth Interneational Symposium on Drug Analysis, September 1995, Leuven, Belgium.

SSDI 0731-7085(95)01706-2

length because of their similar charge to mass ratios. Capillary gel electrophoresis (CGE), which employs permanent gels, is the most widely used technique for analyzing antisense oligonucleotides [5]. A problem is encountered, however, due to the high viscosity of these buffered gels, which require manual filling of the capillary and show instability after prolonged use.

Recently, CE using entangled polymer solutions has gained importance for the separation of DNA molecules [6,7]. In this technique, oligonucleotides can be separated using a dilute polymer solution that can be loaded into the capillary after each run, thus refreshing the separation matrix before every analysis and preventing any sample material from remaining [8]. Micellar electrokinetic capillary chromatography (MECC) has also been used in the past to resolve oligonucleotides with a buffer containing sodium dodecyl sulfate (SDS) and metal ions [9].

The intention of this study was to explore the separation of short model oligonucleotides using MECC and CE in entangled polymer solution and to compare the two techniques on the basis of qualitative and quantitative results.

2. Experimental

Oligonucleotide samples $p(dA)_{21-18}$, $p(dA)_{19-24}$ and $p(dA)_{21}$ (the last used as an internal standard) were purchased from Pharmacia Biotech (Roosendaal, Netherlands). All samples were kept in a freezer (−28°C). All reagents used were of analytical grade. SDS and Tris (tris(hydroxymethyl)aminomethane) were obtained from Acros Chimica (Geel, Belgium). Copper(II) sulfate and disodium hydrogenphosphate were obtained from Merck (Darmstadt, Germany). TAPS (*N*-tris(hydroxymethyl)-3-aminopropanesulfonic acid) and urea were purchased from Sigma (St. Louis, MO) and UCB (Brussels, Belgium), respectively. Hydroxyethylcellulose (HEC EP09) (>86.2% purity) was kindly donated by Union Carbide (Antwerp, Belgium). Milli-Q water (Millipore, Milford, MA) was used throughout. Stock standard solutions of oligonucleotides samples were prepared in Milli-Q water as follows: $p(dA)_{12-18}$ 0.5, $p(dA)_{19-24}$ 0.1 and $p(dA)_{21}$ 0.93 mg ml^{-1}.

CE separation was performed on a Spectra PHORESIS 1000 system (Thermo Separation Products, Fremont, CA), which was driven by CE software (version 1.04) operating under IBM OS/2 (version 1.2). For MECC, fused-silica tubing (Polymicro Technologies, Phoenix, AZ) of 50 μm i.d. with 44 cm total length (L) and 36 cm effective length (l) was used. The capillary was washed for 5 min with 0.1 M NaOH, water and electrolyte before each run. The running buffer used contained 20 mM TAPS, 70 mM SDS, 0.3 mM copper(II) sulfate and 7 M urea (pH 7.7, adjusted with Tris). The oligonucleotide samples were introduced hydrodynamically (0.3 s for qualitative work and 5 s for quantitation) into the capillary filled with running buffer and analyzed at 10 kV (227 V cm^{-1}, 12–13 μA) at 25°C. Methanol was used as a neutral marker and quinine hydrochloride was used as a tracer.

CE separation in entangled polymer solutions was carried out using a J&W DB-17 capillary (50% methylsilicone, 50% phenylsilicone coating) (100 μm i.d. ×44 cm (L) and 36 cm (l)), which was purchased from Alltech (Laarne, Belgium). The capillary was cleaned at the beginning of the day with HPLC-grade methanol for 5 min followed by a water wash for 5 min. The prewash time was 5 min and the capillary was filled with fresh buffer before every analysis. The running buffer consisted of 20 mM TAPS, 4% HEC EP09 and 7 M urea (pH 7.0, adjusted with Tris). The oligonucleotide samples were either introduced electrophoretically at negative polarity (5 kV for 1 s) or hydrodynamically (30 s) into the capillary and separated at a negative voltage of 12 kV (273 V cm^{-1}, 17–18 μA) at 25°C. Oligonucleotides were detected at 260 nm.

3. Results and discussion

3.1. MECC separation of oligonucleotides

The repeatability of electroosmotic flow (EOF) is essential for separation repeatability. A problem of reducing EOF was encountered owing to

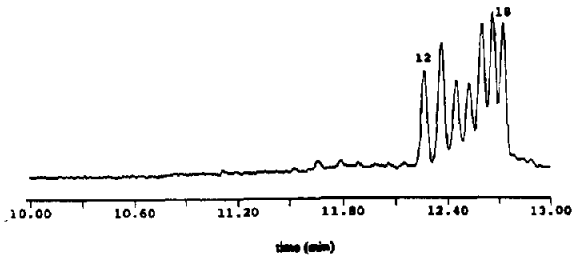

Fig. 1. Separation of $p(dA)_{12-18}$ using MECC. Electrolyte, 70 mM SDS, 0.3 mM copper(II) sulfate, 7 M urea and 20 mM TAPS, adjusted to pH 7.7 with Tris; capillary, fused silica (50 μm i.d. $\times$44 cm (L) and 36 cm (l); temperature, 25°C; voltage, 10 kV; injection, hydrodynamic (0.3 s); detection, UV at 260 nm.

the deposition of urea (evaporation) on the outer surface of the electrode. This happens when the electrode leaves the vial and is wiped off by the vial cap. This reduction in EOF can be avoided by not using the caps, but one can restore EOF by simply cleaning the electrode surface. It was also found to be necessary to use fresh buffer (in our case after 5–6 runs) in order to avoid a shift in EOF and migration time and hence selectivity. In addition, in cases where EOF was normal but a shift in the migration time of the tracer was observed, the capilary was washed with EDTA solution to remove residual Cu(II) ions.

Fig. 1 shows the separation of $p(dA)_{12-18}$ by MECC. This separation was achieved using a fused-silica capillary. Three different capillary internal diameters (30, 50 and 75 μm) were tried in this study. The separation could not be achieved using 75 μm i.d. capillary, whereas 50 and 30 μm i.d. capillaries showed comparable separations. A

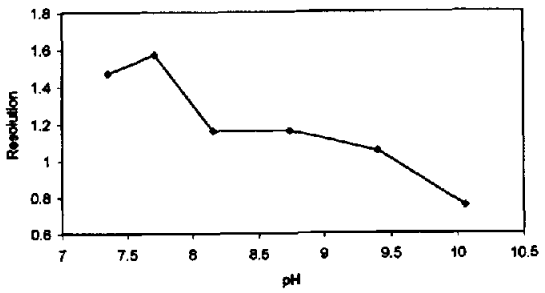

Fig. 2. Effect of pH on the resolution of $p(dA)_{14-15}$. Experimental conditions as in Fig. 1 except for the concentration of SDS (80 mM) and the applied voltage (20 kV).

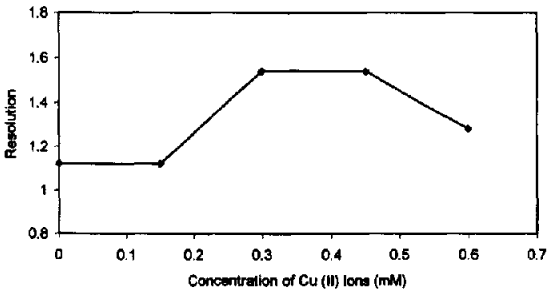

Fig. 3. Influence of concentration of Cu(II) on the resolution of $p(dA)_{14-15}$. Experimental conditions as in Fig. 2.

50 μm i.d. capillary was chosen because of its longer detection path.

We evaluated the pH of the running buffer for the range 7.35–10.06 (six points investigated). The influence of pH on the resolution of the pair $p(dA)_{14-15}$ is presented in Fig. 2. It was seen that a decrease in pH increases the resolution to a certain point. pH 7.7 gave best results in terms of resolution. The influence of the concentration of copper(II) sulfate was studied (0.0, 0.15, 0.3, 0.45 and 0.6 mM). A low concentration (0.3 mM) of copper(II) sulfate was used in the buffer as it was found to increase the retention window and hence the resolution (see Fig. 3) [9]. The effects of the concentrations of SDS (60–90 mM) and TAPS buffer (10–40 mM) were also studied. No change in the resolution values was seen. Similarly, no improvement in the separation was observed with variation in the applied voltage (8–20 kV) and temperature (25–30°C).

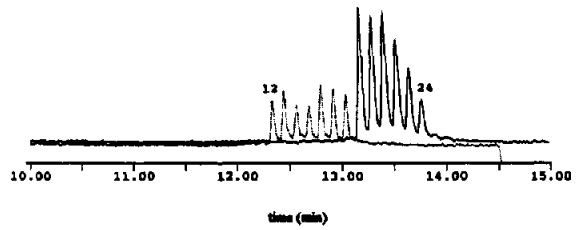

Fig. 4. Separation of $p(dA)_{12-18}$ and $p(dA)_{19-24}$ using entangled polymer solution. Electrolyte, 20 mM TAPS, 4% HEC EP09 and 7 M urea (adjusted to pH 7.0 with Tris); capillary, J&W DB-17 (100 μm i.d. $\times$44 cm (L) and 36 cm (l); temperature, 25°C; voltage, 12 kV (at reversed polarity); injection, hydrodynamic (30 s); detection, UV at 260 nm.

Table 1
Comparison of quantitation results for MECC and CE in entangled polymer solution

Parameter	MECC	CE in entangled polymer solutions
Efficiency, $p(dA)_{13}$	7.6×10^5 m^{-1}	7.3×10^5 m^{-1}
Within-day repeatability ($n = 6$):		
240 μg ml^{-1} $p(dA)_{12-18}$ containing $p(dA)_{21}$ (58 μg ml^{-1}) as internal standard:		
Migration time	RSD[a] 0.04%	RSD 0.2%
Corrected area	RSD 2.6%	RSD 1.5%
Day-to-day repeatability ($n = 4$):		
240 μg ml^{-1} $p(dA)_{12-18}$ containing $p(dA)_{21}$ (58 μg ml^{-1}) as internal standard:		
Migration time	RSD 0.2%	RSD 0.1%
Corrected area	RSD 13.0%	RSD 3.4%
Linearity:15–240 μg ml^{-1} $p(dA)_{12-18}$ containing $p(dA)_{21}$ (58 μg ml^{-1} as internal standard) number of concentrations examined = 5 ($n = 15$)	$y = 0.0015x + 0.0161$[b] ($r = 0.9975$)	$y = 0.0022x + 0.0041$[b] ($r = 0.9985$)
LOD (S/N = 3)[c] $p(dA)_{12-18}$, 3.25 μg ml^{-1}	29 pg (hydrodynamic, 5 s)	31 pg (hydrodynamic, 30 s)

[a] All RSD values were calculated for $p(dA)_{13}$.
[b] Where $y = p(dA)_{13}/p(dA)_{21}$ area ratio and x = total concentration of $p(dA)_{12-18}$.
[c] LOD = limit of detection; S/N = signal-to-noise ratio.

3.2. CE separation of oligonucleotides in entangled polymer solutions

Fig. 4 shows the separation of $p(dA)_{12-18}$ and $p(dA)_{19-24}$ performed with the J&W DB-17-coated capillary using 4% of HEC EP09 at 12 kV. As can be seen, oligonucleotides ranging from 12 to 24 bases in length were baseline resolved. We chose a low-viscosity grade of polymer (HEC EP09) because it could be used at higher concentrations without having problems of replacing the buffer automatically.

3.3. Comparison of MECC and CE in entangled polymer solutions

The comparison of the separation of oligonucleotides (12–24 mer) using a micellar system and sieving buffer led to the following conclusions. Although a micellar electrolyte using a fused-silica capillary is more suited for routine work on the automatic CE equipment used here, MECC is not suitable for separating oligonucleotides more than 13 bases in length. On the other hand, CE in entangled polymer solution has shown the separation of $p(dA)_{12-18}$ and $p(dA)_{19-24}$ with a difference of a single base unit. A comparison of quantitative results for these two techniques is presented in Table 1. Comparable figures for efficiency, linearity and sensitivity were obtained. However, better within-day and between-day repeatabilities were observed for CE in entangled polymer solution.

References

[1] C.A. Stein and Y.-C. Cheng, Science, 261 (1993) 1004–1012.
[2] P.J. Oefner and G.K. Bonn, Int. Lab., July/August (1994) 14–23.
[3] W.J. Warren and G. Vella, Biotechniques, 14 (1993) 598–606.
[4] D. Rickwood and B.B. Hames, in Gel Electrophoresis of Nucleic Acids: A Practical Approach, IRL, Oxford, 2nd edn., 1990, pp. 125–149.
[5] M. Vilenchik, A. Belenky and A.S. Cohen, J. Chromatogr. A, 663 (1994) 105–113.
[6] A.E. Barron, D.S. Soane and H.W. Blanch, J. Chromatogr. A, 652 (1993) 3–16.
[7] B.R. McCord, J.M. Jung and E.A. Holleran, J. Liq. Chromatogr., 16 (1993) 1963–1981.

[8] A.E. Barron, H.W. Blanch and D.S. Soane, Electrophoresis, 15 (1994) 597–615.

[9] A.S. Cohen, S. Terabe, J.A. Smith and B.L. Karger, Anal. Chem., 59 (1987) 1021-1027.