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Determination of single-stranded oligodeoxynucleotides by capillary gel electrophoresis with laser induced fluorescence and on column derivatization

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Abstract

OliGreen™ reagent, a new dye for complexing of oligonucleotides of low molecular mass was successfully utilized for their quantitative analysis using on column derivatization and capillary gel electrophoresis with laser induced fluorescence. Method optimization for the precise, accurate and reproducible quantitation of low-molecular-mass oligonucleotides from aqueous matrices is described.

Keywords: Derivatization, electrophoresis; OliGreen™ reagent; Oligonucleotides; Dyes

1. Introduction

Oligonucleotides (referred in the text as oligos) are being targeted as candidates for a new class of therapeutic agents. Complimentary sequences of these compounds have demonstrated to have high affinity constants with high specificity for mRNA resulting in the inhibition of protein expression [1–3]. In addition, these compounds have also been known to bind to double-stranded DNA potentially halting in vivo transcription [4]. The rational use of these compounds in humans and/or animals and their stability in pharmaceutical dosage forms requires the development of highly sensitive, specific and reproducible analytical methodology. In addition, this methodology can be extrapolated to the detection of failure sequences during the synthesis of oligos. High-pressure chromatography with UV detection has been used for the quantitative determination of these compounds in aqueous matrices [5]. However, capillary electrophoresis with laser induced fluorescence appears to be a better candidate for improved specificity and definitely better sensitivity. The objective of this research is to report the development of a new and improved capillary gel electrophoresis (CGE) method with laser induced fluorescence (LIF) detection for the determination of oligos of low molecular weight using OliGreen™ reagent as the on column derivatization agent. CGE has been recognized as a powerful tool in the separation, purification and sequencing of single (ss), and double (ds) stranded DNA. CGE of DNA fragments usually employs linear or crosslinked polyacrylamide or agarose as a sieving matrix [6–11]. ds-DNA fragments have been successfully separated using replaceable and non-replaceable linear polyacrylamide matrices of low concentration and viscosity [12]. The use of replaceable matrices has

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the advantages that after each electrophoretic run the matrix can be replaced. In addition, replaceable gel matrices allow for the use of the hydrodynamic injection as the choice for the introduction of the analyte into the capillary column. High resolution CGE separations of 3×10^7 plates/m have been reported in the literature [13]. CGE lends itself to automation allowing the processing of large number of samples with high resolution, efficiency and rapid separation times. The introduction of an internal standard and reduction of the ionic strength of the sample improves the precision of electrokinetic injections [14].

The majority of the research conducted on the use of CGE for the analyses of oligos makes use of DNA absorption at 260 nm as the main mode of detection [7,9,14–17]. Laser-induced fluorescence (LIF) detection is known to allow high sensitivity detection. LIF detection of ds-DNA fragments is accomplished by the use of intercalating dyes. CGE analysis of polymerase chain reaction (PCR) samples was performed with the LIF detection using thiazole orange as a fluorescent intercalator [18]. The sensitivity was found to be 100 times that obtained with UV detection. CGE with LIF detection is widely employed in DNA sequencing. LIF detection of tetramethylrhodamine isothiocyanate-labelled DNA fragments allowed for 2 zmol detection limit of the labelled primer [19]. A detection system based on a single laser and two photomultiplier tubes has been used to sequence DNA primers labelled with two fluorescent dyes FAM and JOE [20]. CGE with LIF detection of oligos and DNA sequencing reaction products labelled with fluorescein was reported by Swerdlow and Gesteland [6]. Oligo labelling with fluorescein allows for the use of LIF detection. However, it requires an additional step in sample handling, and presents an obstacle when in vivo metabolic and distribution studies are performed.

In this paper we report a new method for ss-oligo on-column derivatization for their quantitative analysis by CGE with LIF detection. OliGreen™ (referred to in text as dye) is a novel dye for the fluorescent quantitation of ss-DNA at 485 nm and 520 nm emission and excitation wavelengths, respectively. High affinity, specificity and almost instantaneous formation of the fluorescent dye–oligo complex (referred to in the text as complex) makes the dye very valuable for the detection and quantitative analysis of these compounds by CGE with LIF detection.

2. Experimental

2.1. Chemicals and reagents

Acrylamide and Tris were obtained from Pharmacia Biotech (Piscataway, NJ, USA). Ultrapure urea was obtained from ICN Biomedical (Aurora, OH, USA). Ammonium persulfate was purchased from Life Technologies (Gaithersburg, MD, USA). N,N,N′N′-tetramethylethylenediamine (TEMED) was purchased from Fluka (Ronkokoma, NY, USA). Sulfuric and boric acid were reagent grade obtained from J.T. Baker (Philipsburg, NJ, USA). Methanol, reagent grade was purchased from EM Science (Gibbstown, NJ, USA). Methacryloxypropyltrimethoxysilane was obtained from Sigma (St.Louis, MO, USA). Filters (0.2 μm) were purchased from Baxter (McGaw Park, IL, USA). Oligo (15-mer GGTTGGTGTGGTTGG) was a generous gift from Gilead Sciences (Foster City, CA, USA). 20/40 pdT standard was obtained from Beckman (Fullerton, CA, USA). OliGreen™ reagent was obtained from Molecular Probes Inc. (Portland, OR, USA).

2.2. Instrumentation and equipment

Capillary electrophoresis was performed on a PACE 5010 equipped with an argon-ion laser (Laser Module 488, Beckman). The separation runs were performed at an applied voltage of 13 kV at 30°C. The running buffer was 100 m*M* Tris–borate with or without 7 *M* urea. The sample was introduced on column using electrokinetic injection for 10 s at 5 kV. Collected data was analyzed using Beckman's System Gold electrophoresis software (Beckman). Spectrofluorometric studies were performed on the Spex Fluorolog (Industries, Edison, NJ, USA).

2.3. Gel-filled capillaries

Fused-silica capillary tubing I.D. 100 μm and O.D. 375 μm was purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length of the

capillary installed in the CE cartridge is 27 cm with an effective length to the detector window of 20 cm. The tubing is cut to the appropriate length, allowing for 2–3 cm extra on both sides of the capillary to be trimmed before installation into the cartridge. The detection window on the capillary is created by removing the polyimide coating on the tubing using fumes of heated sulfuric acid. The capillary is washed with 1 *M* HCL, followed by a wash with 1 *M* NaOH which is left in the capillary for 1 h. The capillary is then flushed with methanol and treated with 1:1 mixture of methacryloxypropyltrimethoxysilane with methanol. The sililating agent is left in the capillary for at least 3 h at room temperature of 25°C [4]. A solution of 10% linear polyacrylamide (LPA) in 100 m*M* Tris–Borate buffer containing 7 *M* urea is filtered through 0.2-μm filter and degassed under nitrogen for 1 h. Polymerization of 1 ml LPA is initiated by addition of 5 μl of each 10% (w/v) ammonium persulfate and 10% (w/v) TEMED. The resulting solution is introduced in the capillary under nitrogen pressure and left overnight at room temperature of 25°C for complete polymerization with the ends of the capillary immersed in the Tris-borate–urea buffer.

Fig. 1 depicts a useful device assembled for the introduction of reagents into the capillary under nitrogen pressure. The device consists of an Ultra-Torr Cajon Union 1/4" (Arthur Valve and Fitting Co., Austin, TX, USA, catalog # SS-4UT-6) and 1/4" to 1/16" reducer (catalog # SS-100-R4). The reagent to be filled in the capillary is introduced into the glass tube (6×50 mm culture tube #9820, Corning, NY, USA) and fitted into the Cajon Union. The capillary is inserted through the 1/16" side of the reducer and extended into the glass tube. Nitrogen inlet was made perpendicular to the assembly. The capillary is filled under 20 p.s.i. nitrogen back-pressure. The device is convenient, inexpensive and allows for the use of small volumes of reagents. Since LPA polymerization is inhibited by oxygen, its introduction into the capillary under nitrogen allows for optimal polymerization conditions.

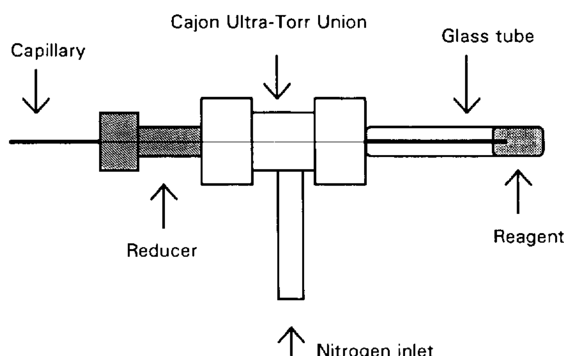

Fig. 1. Device for the introduction of reagents into the capillary under nitrogen pressure.

2.4. *Spectrofluorometric studies*

The dilutions of the dye (1:200 and 1:400) in the Tris–borate buffer with 0 to 7 *M* urea are prepared immediately prior to the experiment. The oligo is added to a cuvette containing 2 ml of the diluted dye to make a final concentration of 1.52 μg/ml. Fluorescence of the final solution is monitored over a period of up to 2 h at 485 nm and 520 nm emission and excitation wavelengths, respectively.

3. Results and discussion

3.1. *OliGreen™ reagent*

OliGreen™ dye was recently introduced as a novel reagent for the quantitation of ss-DNA fragments and small-molecular-mass oligos. Sensitivities in excess of 10 000-fold compared with UV absorbance have been achieved with a standard spectrofluorometer [21]. The dye possesses minimal fluorescence in the unbound form. The fluorescent complex between the dye and the oligo is formed instantaneously in aqueous solutions. This property was exploited in our laboratory for the on-column derivatization of oligos for their detection using LIF.

Electrophoresis of ss-DNA is performed using "reverse" polarity. In the reverse polarity mode the analyte is injected at the cathode with the separation proceeding in the direction of the anode. The dye possesses an overall positive charge. Thus, the vial containing the buffer solution of the dye is positioned at the anode (outlet). The negative side of the capillary (the inlet) is immersed in the running buffer. The column is allowed to equilibrate with the dye by applying the voltage and monitoring the

baseline. A positive shift in the baseline indicates that the dye has reached the detection window. Knowing that the total capillary length is 27 cm and the time it takes for the dye to reach the detection window, i.e., to travel 7 cm, it is possible to estimate the time required for the complete column equilibration with the dye. The analyte is introduced on the column using electrokinetic injection.

CGE separation using "reverse polarity" requires an analyte to be negatively charged. Lack of endoosmotic flow in the gel-filled capillaries will make it impossible for the neutral complex to move along the capillary under the applied voltage. Since the oligo and the dye have charges of opposite polarity the possibility of formation of a neutral fluorescent complex was of concern in the use of the dye as an on-column derivatization agent. LIF detection of well resolved oligos under the conditions of reverse polarity indicates that the derivatized oligos still possess an overall negative charge (Fig. 2).

3.2. Effect of denaturants on the oligo–OliGreen™ complex fluorescence

Assurance of adequate separation and detection of well resolved oligo peaks during the CGE runs required the addition of 7 *M* urea as a denaturant to the Tris–borate running buffer. The same buffer was used to prepare LPA-gel filled capillary columns. Lack of the denaturant in the system results in the detection of broad, poorly resolved oligo peaks. Since the dye is a cationic molecule the effect of such strong denaturant as urea on its complexing properties was investigated. Spectrofluorometric studies were conducted to study the kinetics of the fluorescent complex formation. The results of the study indicate that the fluorescent complex is formed almost instantaneously. However, maximum fluorescence is reached 5 min post-derivatization (Fig. 3). These results indicate that if reliable and reproducible quantitation of oligos with the dye using CE is attempted, the fluorescence measurement should be performed no earlier than 5 min into the separation run.

The results of the experiment also showed, that 7 *M* urea drastically decreases the fluorescence of the oligo–dye complex compared to that in Tris–borate buffer without urea. Increasing concentration of urea in the buffer caused an exponential decay in the fluorescence, with the presence of 1 *M* urea having a minimal effect (Fig. 3). Thus, the presence of urea in the CE separation buffer will drastically decrease the sensitivity of the assay.

The recommended dilution of the dye is 1:200. On-column oligo derivatization requires saturation of the capillary column with the dye. We have investigated the use of higher dye dilution for the quantitation of the oligos. A spectrofluorometric study of the kinetics of fluorescent complex formation using 1:200 and 1:400 dilutions of the dye in the Tris–borate buffer containing 0 and 7 *M* urea was performed (Fig. 4). The results of the experiments indicate that the fluorescent complex is formed instantaneously, i.e., complex fluorescence is detected immediately upon the initiation of the complexation reaction. In the absence of urea, increase in the dilution of the dye results in proportional decrease of the fluorescence of the complex, i.e., the magnitude of the fluorescence at 1:400 dilution is half of that observed with 1:200 dilution. The presence of 7 *M* urea in the system not only suppresses the complex fluorescence, but also changes the proportionality between the dye dilution and the resulting fluorescence signal. In the presence of 7 *M* urea, a 4.5-fold decrease in fluorescence was observed for the 1:400 dilution compared to that at 1:200 dilution, suggesting that any variation of the dye concentration on the column in the presence of 7 *M* urea during the CE separation runs could translate into drastic changes of the height and area of the detected oligo peaks, increasing the variability of the method. Fig. 5 summarizes the kinetics of the fluorescent complex formation at a 1:400 dilution. The fluorescence of the complex appears to be less stable and to gradually decline as a function of time. The decline in fluorescence signal was made more apparent by changing the scales, as illustrated in Fig. 5.

Briefly, from the spectrofluorometric studies it was concluded, that: (1) with respect to the magnitude of the fluorescence response as well as its stability over time a 1:200 dilution of the dye is optimal for the qualitative CE assay; (2) if reliable and reproducible oligo quantitation using the dye is to be attempted, careful attention should be given to the concentration of urea in the CE system.

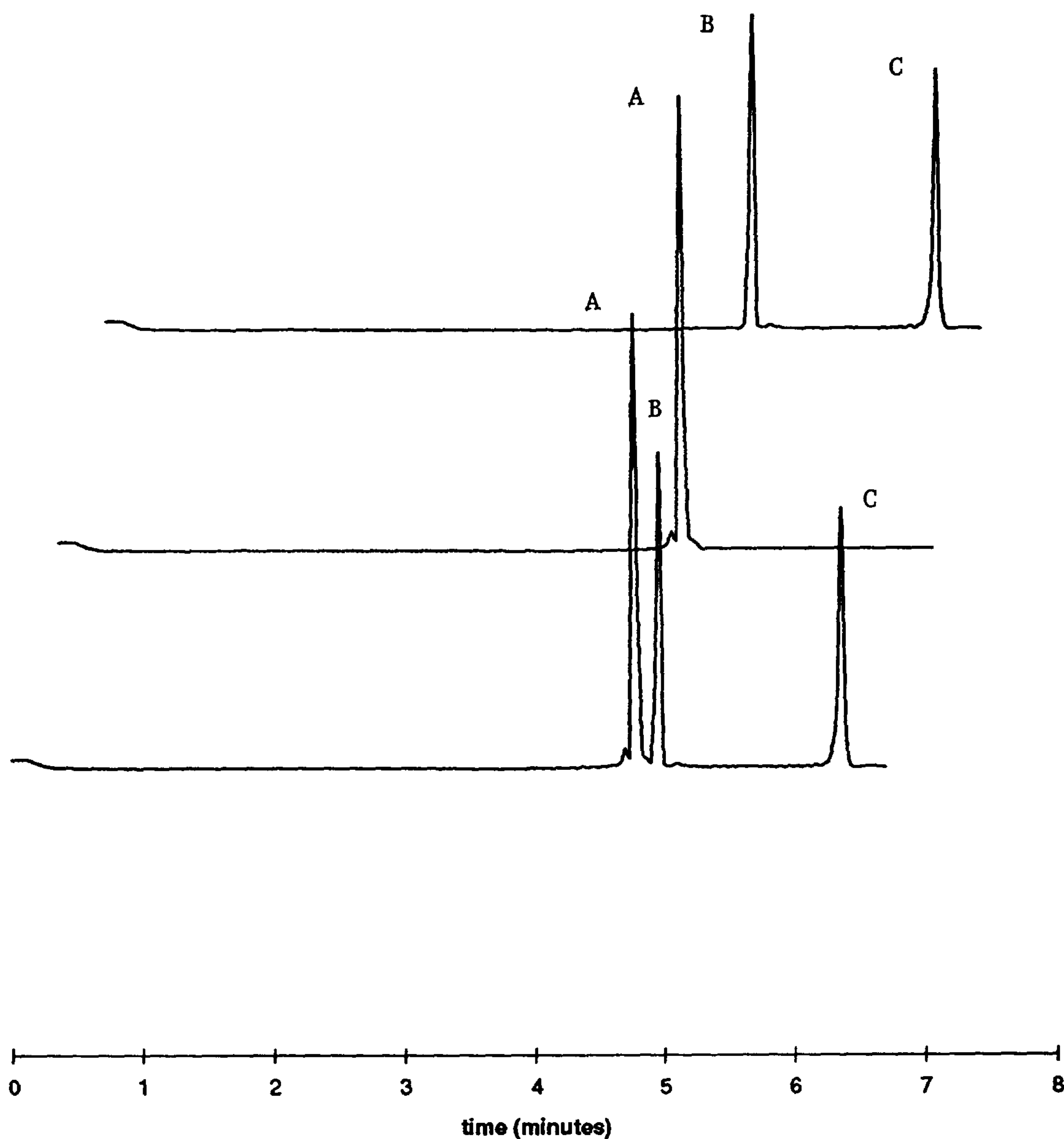

Fig. 2. Electropherograms of the oligo with 20/40 pd(T) internal standard. Peaks A, B, C, are 15-mer, 20-mer and 40-mer, respectively. Conditions: LIF detection, 100 m*M* Tris–borate, no urea buffer, OliGreen™ (1:200 dilution), 13 kV applied voltage, 10 s injection. Oligo concentration 285 ng/ml.

In the search of oligo denaturants that could be used to substitute urea, the effect of formamide and guanidinium thiocyanate as buffer components on the fluorescence of the oligo–dye complex were investigated. No fluorescence above that of a baseline was observed in the presence of 4 *M* guanidinium thiocyanate in the Tris–borate buffer. The magnitude of the fluorescence observed in the presence of 30% formamide was almost negligible.

In the absence of denaturants well resolved oligo peaks can be obtained by increasing the capillary column temperature from 30 to 50°C. However,

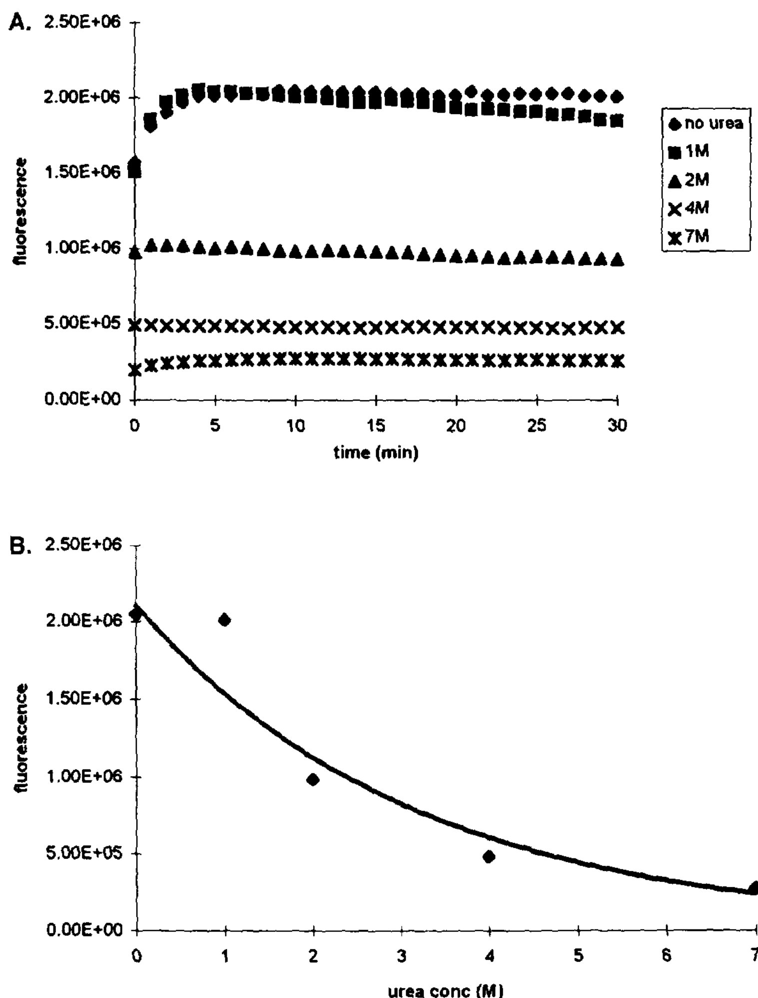

Fig. 3. Effect of different urea concentrations in the Tris–borate buffer on the fluorescence of the oligo–dye complex; 1:200 dye dilution; 1.52 μg/ml oligo concentration. Complex fluorescence was corrected for the dye fluorescence in the unbound state.

elevated temperature is detrimental to the gel matrix and cause drastic decrease in the lifetime of the capillary. High temperatures are also harmful to the stability of the dye.

OliGreen™ reagent is supplied in a dimethylsulfoxide (DMSO) stock solution. DMSO is known to possess denaturant properties. Since DMSO is not detrimental to the dye, it was thought that it could be used as urea substitute in the CE running buffer. However, the incorporation of 50% DMSO into the Tris–borate buffer was highly detrimental to the LPA capillary column. Current deterioration was observed immediately after voltage application to the column, making the capillary column unusable.

It was determined that the presence of urea in the CE system decreases the sensitivity and increases variability of the method. However, since none of the tested denaturants proved to be useful, different concentrations of urea in the buffer and in the capillary were tested to determine the lowest urea concentration required in the system to provide optimum denaturating conditions for the oligos. LPA capillaries with no urea were prepared, and a set of Tris–borate running buffers with 0, 2, 4 and 6 *M*

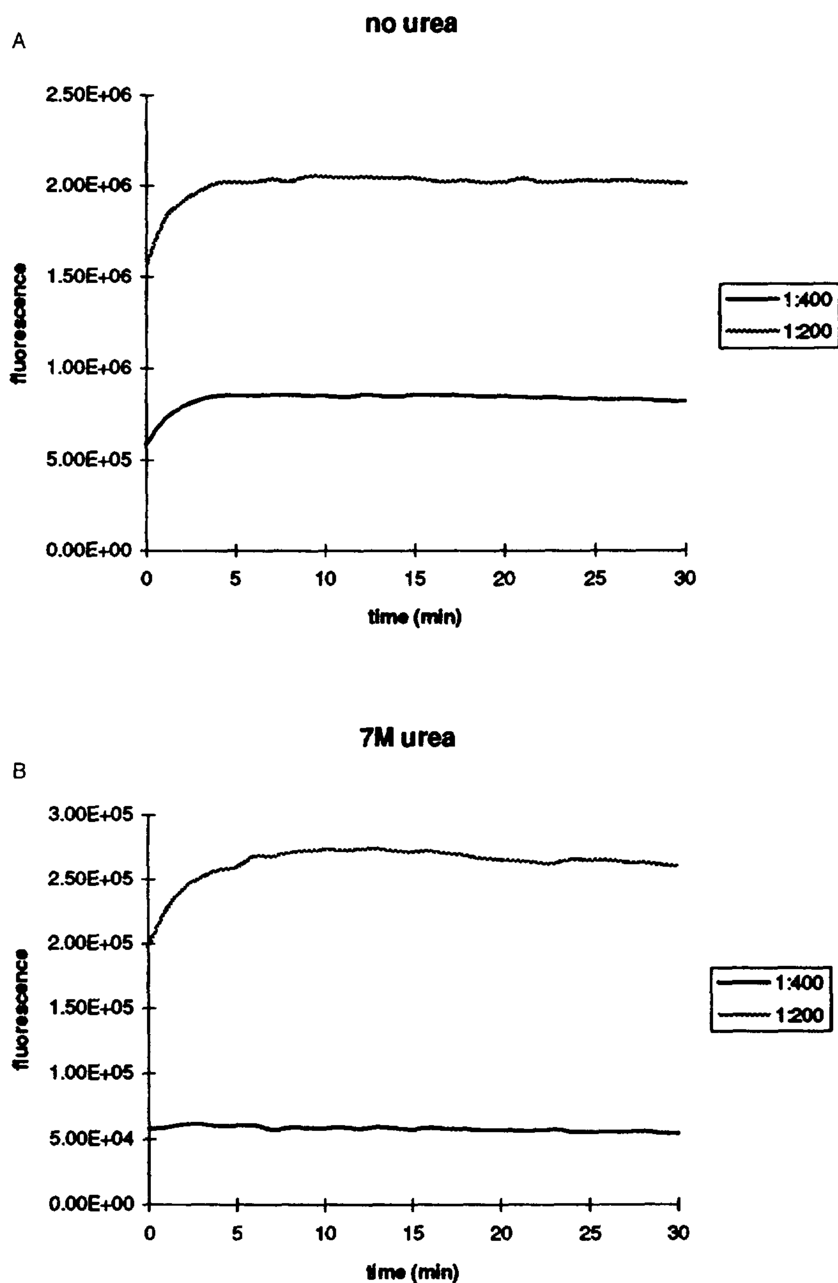

Fig. 4. Dependence of the fluorescence of the oligo–dye complex on the dye dilution; (A) no urea and (B) in the presence of 7 *M* urea in the Tris–borate buffer; 1.52 μg/ml oligo concentration. Complex fluorescence was corrected for the dye fluorescence in the unbound state.

urea concentrations were made. Under these conditions oligo peaks were wide and poorly resolved. Thus, it was concluded that urea in the capillary matrix is necessary to ensure proper oligo denaturation. Four (4) molar urea in the capillary matrix was found to yield reproducible and highly resolved oligo

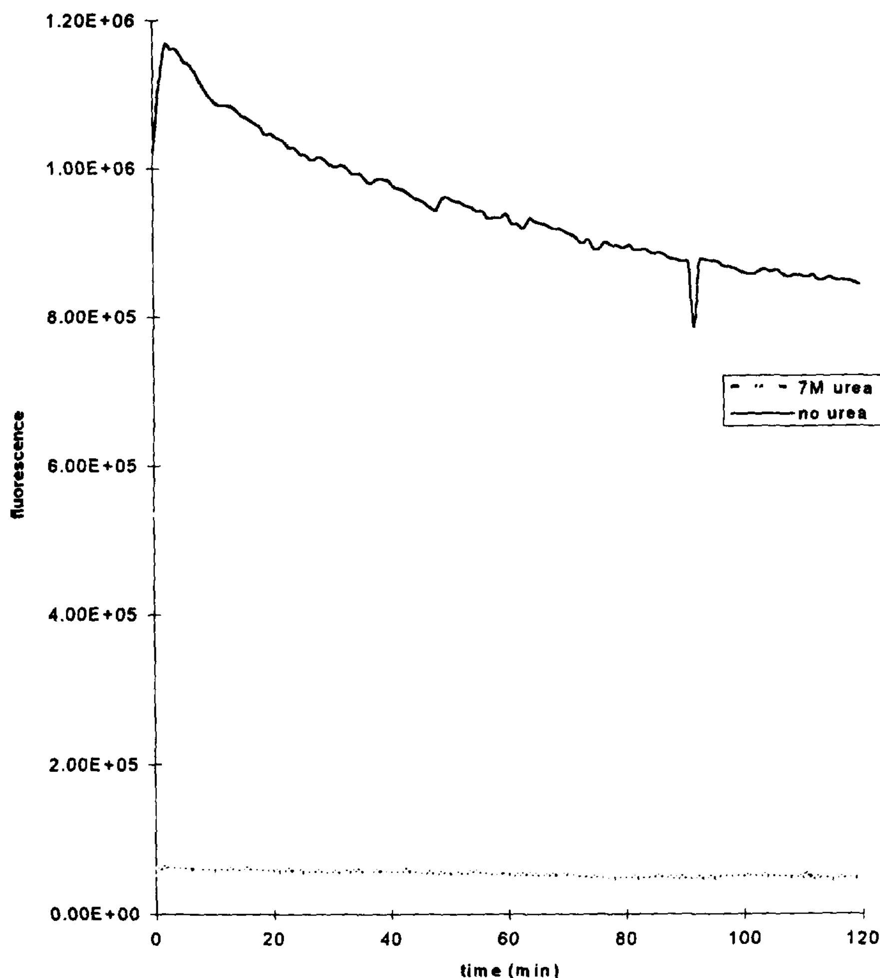

Fig. 5. Effect of 7 *M* urea in Tris–borate buffer on the stability and fluorescence of the oligo–dye complex at 1:400 dye dilution; 1.52 μg/ml oligo concentration. Complex fluorescence was corrected for the dye fluorescence in the unbound state.

peaks using Tris–borate with no urea as the running buffer. This system was comparable in peformance with that of 7 *M* urea in both the capillary matrix and the running buffer. These conditions were accepted as a compromise between the denaturing function of the urea and its possible contribution to the method variability.

3.3. Electrokinetic injection

Capillary gel electrophoresis performed on the gel-filled capillary columns employs electrokinetic injection as the only mode of the analyte introduction on the column. Electrokinetic injection is based on the same principle as electrophoresis. Application of a voltage potential of short duration to the sample solution forces the analyte ions to be electrophoretically drawn into the capillary. The presence of charges other than those of the analyte in the sample can result in the biased injection due to the interaction of charges. The possibility of a biased injection is a major concern for its use in the analyte quantitation. A mixture of 20 and 40-mer pd(T) was used as an internal standard to minimize the variability due to the injection. Injection reproducibility of the UV and LIF modes of detection was found to be 2.28% (2.21±0.05) and 6% (4.56±0.27), respectively. Increased coefficient of variation of the LIF

detection compared to that of the UV detection might be attributed to complexing reaction.

3.4. Migration time reproducibility

Migration time reproducibility of the electrokinetic injection using UV and LIF detection of the oligos was found to be 0.23% (6.93 min±0.016) and 0.94% (5.51 min±0.052), respectively. Normalization of the migration time of the analyte by that of the internal standard improves migration time reproducibility 9 times (from 0.23 to 0.026%) for the UV detection and 3.9 times (from 0.94 to 0.24%) for the LIF detection. Overall, migration time reproducibility of oligos on the gel-filled capillaries is satisfactory.

3.5. Quantitation of oligonucletiodes using CE with LIF

Quantitation of the oligos from the aqueous matrix was performed on the gel-filled capillaries prepared using 4 *M* urea Tris–borate buffer. Tris–borate running buffer contained no urea. The analysis time was reduced compared with that previously reported from 35 min to under 9 min by increasing the applied voltage to 13 kV and shortening the capillary length to 27 cm [10]. It was found that lower dye dilution (1:150) compared with the recommended one (1:200) yielded highly reproducible results. However, for qualitative purposes a 1:200 dilution of the dye is still adequate.

The linear dynamic range of the detector response was found to be between 0.01 and 1.5 μg/ml (R_{sq}= 0.998). Accuracy of the method ranges from 2.5–5.3% for the 190 ng/ml, 769 ng/ml and 1.52 μg/ml samples. Precision of the 1.52 μg/ml sample is 5.98%. Intra-day variability for the 760 ng/ml sample was found to be 1.88%. Limit of detection was found to be 1 ng/ml with LIF detection, compared with 30 ng/ml with the UV detection.

4. Conclusions

The results of this work demonstrate that OliGreen™ reagent, a new dye for complexing ss-oligos, was successfully utilized for their quantitative analysis from aqueous matrices. This method can be extended for purity determination of oligos using a 1:200 dilution of dye in the CGE running buffer. The following conditions are recommended to ensure sensitive and reproducible quantitative analysis of ss-oligos: (1) low ionic strength of the sample; (2) lack or minimal urea content in the CGE system; (3) a 1:150 dilution of the dye in the CGE running buffer; (4) fluorescent complex detection no earlier than 5 min post-derivatization. The method presented in this paper may be extrapolated for reliable quantitative analysis of oligos of any sequence provided the above described conditions are met. We are presently investigating the use of this method for the determination of ss-oligos in biological matrices, e.g., human and animal blood plasma.

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