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Journal of Chromatography A, 979 (2002) 307–314

JOURNAL OF CHROMATOGRAPHY A

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Handling and detection of 0.8 amol of a near-infrared cyanine dye by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

We are interested in the detection of DNA adducts and other trace analytes by labeling them with a fluorescent tag followed by use of capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) for high resolution and sensitivity. Towards this goal, here we report the following: (1) synthesis and handling properties of a near-IR, carboxyl-substituted heptamethine cyanine dye; (2) modification of an existing ball lens LIF detector to provide near-LIF detection with excitation at 785 nm for CE; and (3) corresponding handling and detection of as little as 0.8 amol of the dye by enrich-injection of 4.7 μl of $1 \cdot 10^{-13}$ mol/l dye in methanol from an 8-μl volume into a corresponding CE–LIF system. The electrolyte for the separation was methanol–40 mmol/l aqueous sodium borate (98:2, v/v). This finding encourages further exploration of the dye by functionalization of its carboxyl group for chemical labeling purposes.

Keywords: Detection, electrophoresis; Dyes; DNA; Cyanine dyes

1. Introduction

Capillary electrophoresis with laser-induced fluorescence detection (CE–LIF) potentially has attractive features for trace analysis: high sensitivity, high plate numbers, ease of varying selectivity, and ease of cleaning the column between injections. However, aside from measurement of standards or special applications such as DNA sequencing, CE–LIF is not used very much for trace analysis because of a number of problems, in particular the following: (1) few analytes are inherently fluorescent; (2) fluorescent contamination of reagents and surfaces at the trace level is common; and (3) fluorescence derivatization creates background noise. This noise is due to residual fluorescent reagent, fluorescent contaminants in this reagent, and derivatization of sample contaminants with this reagent.

We are interested in advancing the usefulness of CE–LIF for detecting DNA adducts, which requires

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01441-3

remedies for the above problems. In prior work in this area, we have introduced dyes containing an imidazole group to enable specific labeling of nucleotides [1,2] and have shown that submarine gel electrophoresis can be used to highly purify a dye [3]. Part of the advantage of such labeling is that the products can be readily detected by mass spectrometry [4]. We have also developed relatively stable near-IR fluorescent dyes that can be detected with high sensitivity by CE–LIF [5]. Such dyes may be attractive as derivatization reagents since fluorescent contamination in the near-IR region (700–1000 nm) is rare, even in a cationic detergent [6]. Finally, we have developed a practical, modular LIF detector for CE in the UV range based on a ball lens [7].

The low detection limits (DLs) of CE–LIF can be demonstrated in four ways. The first approach may be called ''peak DL'', where the amount of a fluorophore corresponding to the smallest peak is reported. The sensitivity of this approach reaches the detection of single dye molecules [8,9]. Second is ''injection DL'', which reports the lowest amount or concentration of the dye that has been injected and detected, whether actual or extrapolated. For example, Timperman et al. [10] actually injected and detected about 290 molecules of sulforhodamine, and Chen and Dovichi [11] actually injection-detected about 30 molecules of rhodamine 6G ($4.13 \cdot 10^{-11}$ mol/l injected into a capillary having a 10 μm internal diameter). Third is ''handling DL'', which gives the smallest amount of the dye that can be handled and detected. There have hardly been any reports of handling-DL limits for dyes by CE–LIF in the literature. And fourth is ''real sample DL'', where the detectable amount of an analyte (whether inherently fluorescent or requiring derivatization) in a given amount of a real sample is reported. While each type of detection limit is important, the last one of course is most important in the real world.

Towards a goal of achieving high sensitivity by CE–LIF for the analysis of DNA adducts in real samples (fourth kind of detection), we report our progress here in handling a small amount of a near-IR dye. More specifically, we report: (1) extension of our ball lens LIF detector into the near-IR range; (2) synthesis of a stable heptamethine cyanine near-IR dye that contains a carboxyl group for ease of functionalization along with structural features that make it easy to handle in methanol or aqueous methanol; and (3) handling and detection of 0.8 amol of this dye. Other laboratories have also advanced heptamethine cyanine dyes as labeling reagents for trace analysis [6,12,13]. Schmitz et al. have studied the detection of DNA adducts by fluorescence-labeling CE–LIF [14].

2. Experimental

2.1. Dye synthesis

Dye **2** was obtained by reaction [15] of the intermediate compound **1** [6] with 4-mercaptobenzoic acid. Thus, a solution of **1** (1.9 g, 2.7 mmol/l) and 4-mercaptobenzoic acid (1.0 g, 6.5 mmol/l) in *N*,*N*-dimethylformamide (50 ml) under an argon atmosphere was allowed to stand at 23 °C for 2 days, and then the mixture was treated with ethanol–diethyl ether (1:19, 200 ml). The resultant precipitate of **2** was crystallized from 95% ethanol: yield 1.76 g (66%); m.p. >200 °C (decomp.); ^{1}H NMR (400 MHz, [$^{2}H_6$]dimethylsulfoxide) δ 1.39 (s, 12H), 1.93 (m, 2H), 2.01 (m, 4H), 2.58 (m, 4H), 2.82 (m, 2H), 4.35 (m, 4H), 6.55 (d, $J = 14.0$ Hz, 2H), 7.22 (t, $J = 7.6$ Hz, 2H), 7.37 (d, $J = 8.6$ Hz, 2H), 7.39 (d, $J = 7.6$ Hz, 2H), 7.49 (d, $J = 8.6$ Hz, 2H), 7.51 (d, $J = 7.6$ Hz, 2H), 7.86 (d, $J = 8.6$ Hz, 2H), 8.54 (d, $J = 14.0$ Hz, 2H); ^{13}C NMR (100 MHz, [$^{2}H_6$]dimethylsulfoxide) δ 20.5, 23.4, 26.0, 27.2, 42.9, 47.8, 48.7, 102.3, 111.5, 122.4, 125.0, 125.4, 127.8, 128.6, 130.5, 133.6, 141.2, 142.1, 143.0, 144.8, 147.1, 166.7, 172.1.

Analysis. Calculated for $C_{43}H_{47}ClN_2Na_2O_8S_3 \cdot 5H_2O$: C, 52.30; H, 5.82; Cl, 3.59; N, 2.84. Found: C, 52.47; H, 5.56; Cl, 3.32; N, 2.82. As can be seen from the elemental analysis of **2**, the cationic chromophore is neutralized by chloride ion and the sulfonate groups by sodium ions.

2.2. Near-IR laser CE–LIF system

A G1600A CE system (Agilent Technologies, Wilmington, DE, USA) was employed. The near-IR LIF detector introduced here has been commercialized (ZETALIF near-IR LIF detector, Picometrics, Ramonville, France) and uses a patented optical

bench. The excitation wavelength of this detector is provided by a 785 nm Melles Griote diode laser from Power Technology (Mabelvale AR, USA). The laser power is 50 mW and the elliptic laser beam (about 6×3 mm) directly illuminates, without an optical fiber, the 785 optical filter kit. The CE system was set to the CE–MS mode for external detection, using CE–MS cassette G1600-60002 (Agilent) for this purpose. Data acquisition for external detection on the CE system was through a Multichannel Interface (part number 35900E from Agilent). Vial insert 5181-1270 from Agilent was used to handle as little as 3.0 μl for injection.

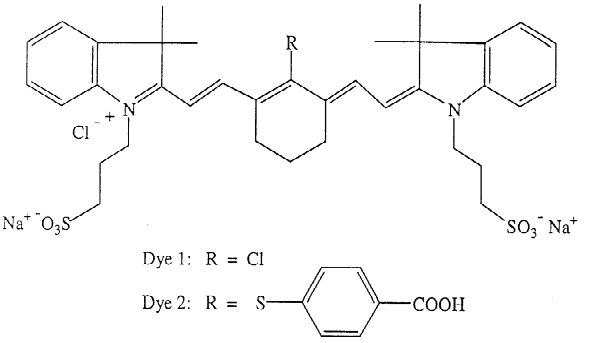

Fig. 1. Structures of dye **1** (synthetic precursor) and dye **2**.

2.3. Capillary electrophoresis

Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with internal diameters of 75 and 100 μm were used. In all cases, capillary length was 75 cm, and the length between the inlet end and the detection window was 60 cm. The separation voltage was set at 30 kV unless otherwise indicated. A pressure of 50 mbar was used to flush the capillary with running buffer for 1 min before each CE run, and for the CE injections. All CE experiments were performed at room temperature. The CE running buffers were prepared without pH adjustment unless indicated otherwise, e.g. 2.5 mmol/l $Na_2B_4O_7$ had a measured pH of 9.2. All injections were made at the anode end of the capillary unless indicated otherwise. Injection volumes were estimated by comparison with times needed under the same conditions for the sample to reach the detector by continuous infusion.

3. Results and discussion

Shown in Fig. 1 is the structure of the heptamethine cyanine dye (dye **2**) that we synthesized from known dye **1**. We chose to establish a carboxyl functional group, as seen for dye **2**, since this group can be readily converted into an active ester for reaction with an amine-substituted analyte or with a linker compound such as histamine to establish an alternative functional group. Previously we employed the latter strategy to make a histamine derivative of a BODIPY dye [3]. The high stability of an amide group makes it attractive as a structural component in tagging reagents. For example, in our earlier studies with a BODIPY dye, we encountered difficulties when a corresponding hydrazide structural component was employed [2].

A schematic of our near-IR LIF detector is shown in Fig. 2. The design is basically the same as that of the UV–LIF we reported previously [7], where an argon ion laser was employed for excitation at 488 nm. Here a diode laser emitting 50 mW at 785 nm is used. A key is the high numerical aperture sapphire ball lens, which directly contacts the capillary and efficiently both delivers the excitation light to the interior diameter of the capillary (over a length of about 200 μm) and collects the fluorescence. The collected fluorescence passes through the dichroic mirror and a series of filters before it is converted into an electrical signal by a photomultiplier tube and treated with a data acquisition system. In our experience, this detector is very rugged as little or no effort is necessary to set it up and keep it in a proper configuration for high sensitivity, including changing of capillaries. No optical alignment is necessary after changing capillaries.

Preliminary experiments with a near-IR dye from Fluka (number 15167) revealed about a 1.4-fold higher sensitivity when a capillary having an internal diameter of 100 μm was used rather than 75 μm. We therefore continued our studies with the former capillary. Injection (3 s, 50 mbar, about 90 nl) of $5 \cdot 10^{-11}$ mol/l dye in running buffer (2.5 mmol/l sodium borate, pH 9.2) into a 100-μm I.D. capillary gave $S/N = 3$ (migration time 4.3 min; data not shown).

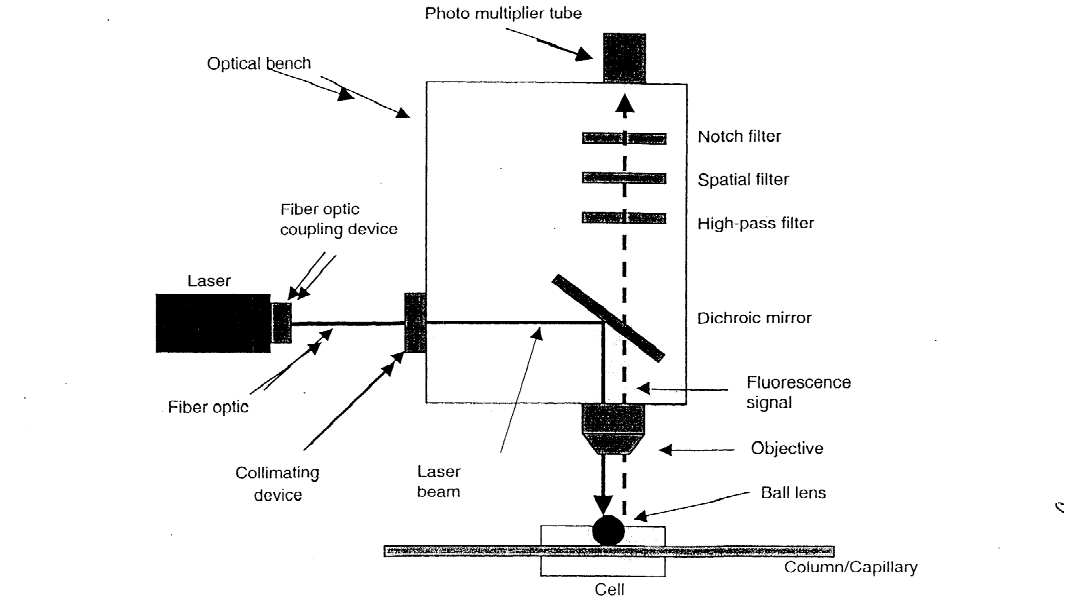

Fig. 2. Schematic of the near-IR LIF detector.

Our first concern with the newly synthesized dye **2** was the best solvent for handling trace amounts of it. From our prior experience, and that of others [17], we anticipated that a polar organic solvent such as methanol would be the best choice, but water is a useful solvent as well for CE, so we decided to study both of these solvents. A stock solution of 10^{-3} mol/l **2** in methanol was diluted to 10^{-9} mol/l in this same solvent, and then an additional dilution was made of 1:100 either into methanol (solution A) or water (solution B). The final dilutions were made into both glass and plastic vials, and the resultant solutions were kept for 1 h at room temperature in the dark before measurement by CE–LIF. The resulting, relative peak heights were: 1.0 (final dilution in methanol into a glass vial), 1.0 (methanol, plastic), 0.40 (water, glass), and 0.11 (water, plastic). Thus, the dye is prone to adsorption losses in water in both plastic and glass containers, and is best handled in methanol irrespective of the container.

In general, methanol as opposed to water has also been a preferred solvent for dyes of this type for two other reasons. The first is the tendency for such dyes, especially the less-polar versions, to self-associate in water [17]. The effect of solvent on the absorption spectrum of **2** in various solvents ranging from 0 to 100% methanol is shown in Fig. 3, and there is no evidence for dimerization of the dye under these

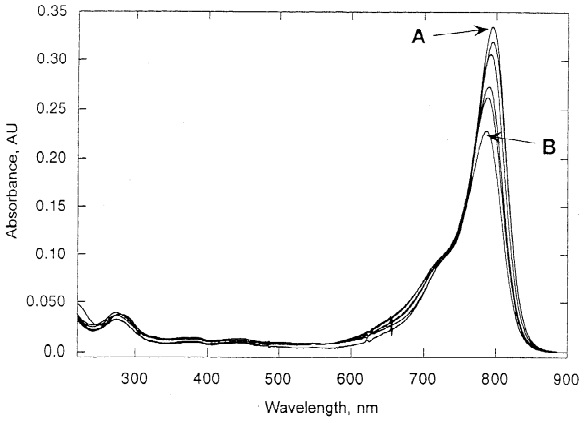

Fig. 3. UV–Vis spectra of dye **2** in various solvents. The solvent composition was varied from pure MeOH (A) to pure water (B) and included 80% methanol, 60% methanol, 40% methanol, 20% methanol and 10% methanol.

conditions, nor did the spectra vary with time. Dimerization of a heptamethine cyanine dye is usually evident by the appearance of absorption peaks at lower or higher wavelengths, dependent upon the dimer configuration. Such dimers also tend to have lower fluorescence quantum yields [16,18].

To further test the handling and spectral properties of **2**, we infused various solutions of $1 \cdot 10^{-9}$ mol/l of this dye into CE–LIF, which gave the data shown in Fig. 4. The capillary was filled with 2.5 mmol/l aqueous sodium borate before infusion, and continued infusion of this same solvent as a blank gave curve F. The data in Fig. 4 may be due to several factors: adsorption loss of dye in the container prior to infusion; adsorption loss of the dye in the capillary; and variation in dye brightness in the different solvents. Nevertheless, we can interpret the data as follows, keeping in mind that the samples were infused immediately upon preparation (dilution), so that the fluorescence values at 60 min correspond most closely to the conditions of the samples in the prior experiment.

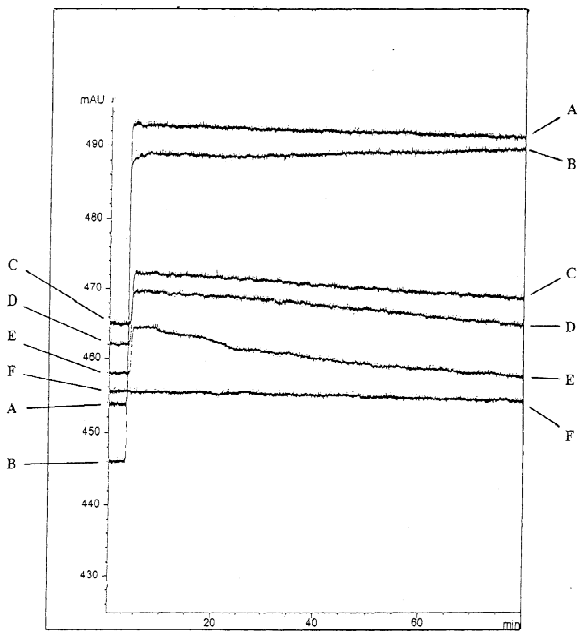

Fig. 4. Infusion of $1 \cdot 10^{-9}$ mol/l dye **2** in: (A) methanol–40 mmol/l aqueous sodium borate (98:2, v/v) from a plastic vial; (B) methanol, from a plastic vial; (C) 2.5 mmol/l sodium borate, from a glass vial; (D) water, from a glass vial; (E) water, from a plastic vial; and (F) running buffer (2.5 mmol/l sodium borate). All curves actually began on a given day at about the same position, and are offset for ease of visualization.

Methanol and methanol–40 mmol/l aqueous sodium borate (98:2, v/v) are the best solvents (curves A and B of Fig. 4, respectively, solutions kept in a plastic vial), since they produce the most intense and stable signals. Curve C, from the infusion of dye in 2.5 mmol/l sodium borate (glass vial), shows about 20% fluorescence relative to A at the outset, and this fluorescence then diminishes slowly over 1 h. Curve D (100% water, glass vial) behaves essentially the same. We interpret the negative slope of these latter two curves as coming from adsorption loss of the aqueous dye with time in the glass vials, consistent with the above observations. The initial ratio of curves C or D to A also is about 20% when 10^{-8} mol/l dye is infused instead of 10^{-9} mol/l. Thus, it can be concluded that dye **2** is about five-fold brighter in methanol than water. Others have observed that a similar dye is brighter at higher percentages of methanol [14], and the quantum yield for a less similar (trimethine) cyanine dye was found to be three times higher in methanol than in water [19]. Curve E (water, plastic vial) slopes negatively most severely, which also is consistent with the above observation that the adsorption loss is greatest when the dye is kept under these conditions.

The above considerations make it clear that methanol solutions may be preferred as both the injection solvent and electrolyte for CE–LIF of **2** in order to enhance the signal-to-noise ratio by minimizing adsorption loss while potentially enhancing dye brightness. Nevertheless, aqueous-based electrolytes offer many opportunities for selectivity in CE, and also for making enrichment injections to enhance sensitivity. As observed above, the brightness of **2** in aqueous CE–LIF is at least 20% of the value in methanol, which is respectable. Accordingly, we decided to study both methanol-rich and aqueous-rich electrolytes for detection of **2** by CE–LIF, but with an emphasis on methanol as the handling and injection solvent.

Others have shown that enrichment injection in CE can be accomplished with samples dissolved in an organic-based solvent [20–22]. For example, Shihabi and coworkers have achieved enrichment of both acidic and basic analytes by using an injection solvent of acetonitrile–150 mmol/l aqueous NaCl in

combination with an aqueous running buffer such as 250 mmol/l sodium borate with a pH near 9.0, or aqueous buffers containing triethanolamine and an organic co-solvent (10% acetonitrile or 20% isopropanol) along with other additives [20,21]. Morales and Cela stacked phenols in nearly 100% acetonitrile as an injection solvent in combination with a nonaqueous running buffer containing ammonium acetate and potassium acetate [22].

We were unable to detect **2** under the enrichment conditions of Shihabi [20,21], or by substituting methanol for acetonitrile. However, it was observed that detection of **2** under enrichment injection conditions could be accomplished by injecting a methanol solution of **2** into 2.5 mmol/l aqueous sodium borate as the electrolyte. Moderate enrichment conditions (5 s at 50 mbar, which injected 0.15 μl) gave the electropherogram in Fig. 5C (inset) from a $1\cdot10^{-9}$ mol/l solution of the dye in methanol, and more intense enrichment conditions (16 s at 50 mbar which injected 0.5 μl) gave the electropherogram in Fig. 5A from a $1\cdot10^{-12}$ mol/l solution. Injection of

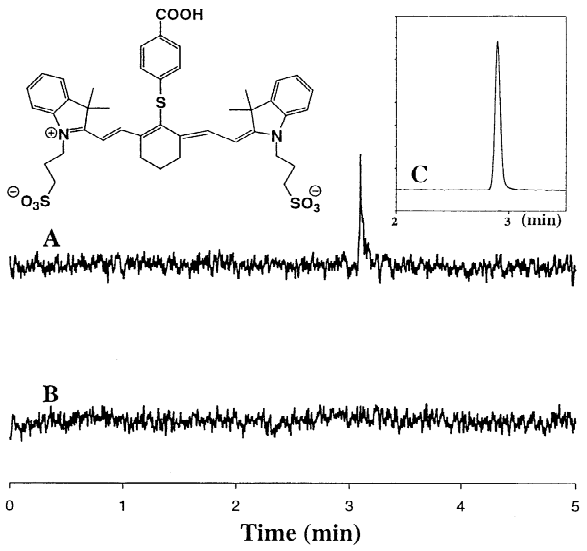

Fig. 5. Near-IR LIF electropherograms: (A) injection (50 mbar, 16 s) of 0.5 μl of dye **2** as a $1\cdot10^{-12}$ mol/l solution in methanol; (B) injection of running buffer (50 mbar, 5 s); (C) injection of dye **2** as a $1\cdot10^{-9}$ mol/l solution in methanol (50 mbar, 5 s). CE conditions: 2.5 mmol/l aqueous sodium borate running buffer (measured pH 9.2); 75 cm (60 cm to the detector)×100 μm capillary; 30 kV. Response axes: arbitrary fluorescence units.

methanol itself as a blank gave electropherogram Fig. 5B. Injection times longer than 64 s at 50 mbar (32% of the capillary volume) gave a split peak. There was no improvement when the running buffer was 10 mmol/l sodium borate, and 40 mmol/l gave erratic results, perhaps due to air bubbles from Joule heating.

We also tested the enrichment injection conditions of Legendre et al. [6], in which the injection solvent is methanol, and the electrolyte is methanol–40 mmol/l sodium borate (98:2, v/v). In principle, the high concentration of methanol in both the injection solvent and electrolyte in this approach should provide the highest sensitivity. Performing the injections at different injection times gave the data shown in Fig. 6A (peak height vs. injection time) and Fig. 6B (migration time vs. injection time). The migration time drops markedly with injection volume, so there is high electroendosmosis in the injection plug (where the capillary is still wetted with pH 9.2 borate buffer). Ordinarily, under equilibrium conditions, a high concentration of methanol lowers electroendosmosis [23]. However, nonequilibrium conditions are present in our experiment, along with a small difference (2%) in methanol concentration between the sample plug and running buffer. We found that peak height increases up to about 150 s of injection. Injecting a $1\cdot10^{-13}$ mol/l solution of the dye in methanol for 160 s (4.7 μl injection volume) gave the electropherogram shown in Fig. 7. To make this injection, 8.0 μl was handled in a microvial insert on the CE–LIF system, so the handling detection limit under these conditions is 0.8 amol. (In other experiments we have injected 0.5 μl from 3.0 μl in the microvial insert.) When 40 mmol/l sodium borate was replaced with 40 mmol/l sodium or potassium carbonate, lower plate numbers were observed, as was the case when 1 or 3% aqueous borate was used instead of 2%. The $1\cdot10^{-13}$ mol/l solution of the dye in methanol was completely stable for at least 2 days when stored in the dark in a plastic vial at −8 °C. Other extended storage conditions were not tested.

As seen in Fig. 7, the plate number is low ($N=$ 3200), and the migration time is short (3.4 min). This would provide very little resolution of DNA adducts. Thus, variations of this condition need to be tested. Such work will commence after we prepare dye–

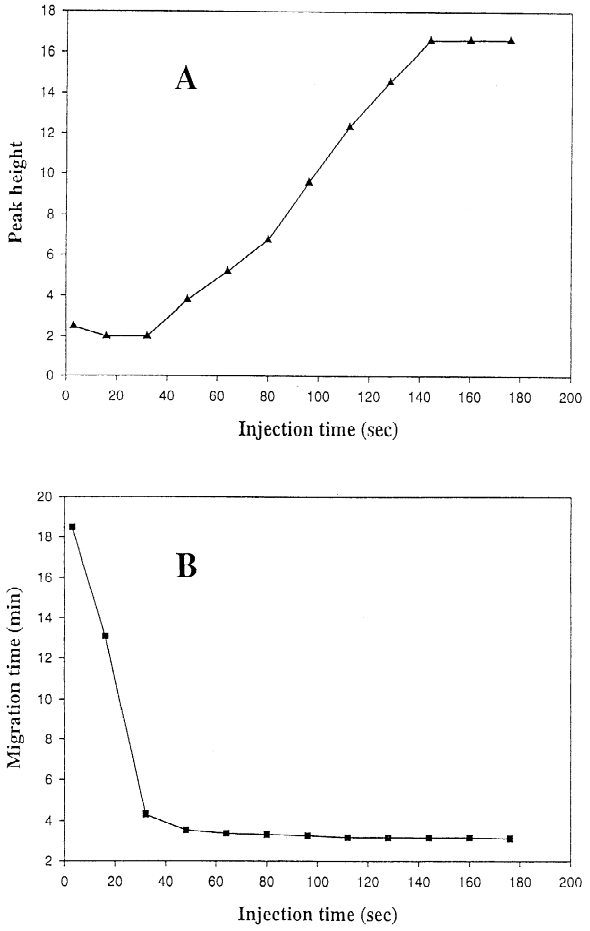

Fig. 6. Peak height (A) and migration time (B) vs. injection time by near-IR CE–LIF for dye **2**: $1 \cdot 10^{-9}$ mol/l in methanol was injected into a running buffer of methanol–40 mmol/l aqueous sodium borate (98:2, v/v).

DNA adduct conjugates to make the selectivity studies meaningful.

4. Conclusion

Both the new near-IR dye and the corresponding new near-IR detector reported here have performed well. This is primarily evidenced by our ability to handle 0.8 amol of the dye for detection by CE–LIF. We therefore plan additional studies of this dye as a tag for derivatization and detection of trace analytes such as DNA adducts.

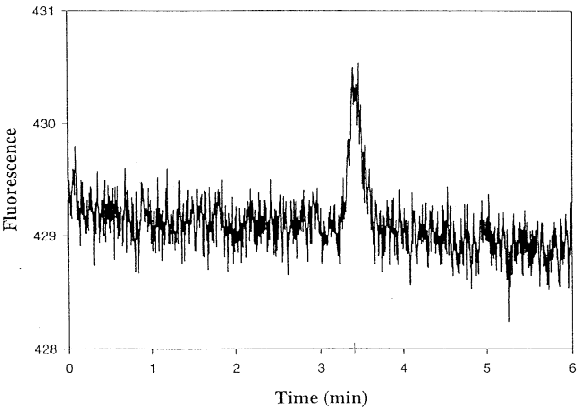

Fig. 7. Detection of $1 \cdot 10^{-13}$ mol/l dye **2** in methanol by near-IR CE–LIF. Injection solvent: methanol (4.7 μl, which is 80% of the capillary volume) was injected from an 8-μl sample by applying 50 mbar for 160 s. Running buffer: methanol–40 mmol/l sodium borate (98:2, v/v). The injection was made at the anode end of the capillary.

Acknowledgements

This work was supported by contract 340811E3119 from the Massachusetts Department of Public Health, and NIEHS grant ES10539. Contribution No. 818 from the Barnett Institute.

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