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Separation of near-infrared fluorescent conjugates of dATP and related compounds by capillary electrophoresis

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Abstract

Capillary electrophoresis was examined as a means for the separation and quantitation of deoxyadenosine triphosphate (dATP) and other nucleotides that were labeled with the near-infrared fluorescent dye IRD700 or related tags. Under the final optimized conditions the labeled dATP was separated from several possible impurities, including the unconjugated forms of IRD700 and dATP, as well as dADP, dAMP and their corresponding IRD700 conjugates. The assay was performed under two sets of conditions. First, the sample was injected onto a 50 cm×75 μm I.D. fused-silica capillary at 25 kV in the presence of a pH 9.5, 140 m*M* borate running buffer. The resulting peaks were monitored at both 254 and 680 nm, where the latter wavelength was used to identify any species that contained the IRD700 label. A second injection was then performed under the same conditions but with a fixed concentration of dTTP now being added to the running buffer; this resulted in the formation of a complex between the dTTP and any dATP, dADP or dAMP-containing components, which changed their rates of migration and allowed them to be differentiated from unconjugated IRD700 or dye contaminants. Only 6 nl of a 1:10 diluted sample were required per analysis. The limit of detection at this injection volume was approximately 1.0 μ*M* (or $6\cdot10^{-15}$ mol for a 6-nl injection) for each monitored component. The linear range extended up to at least 80 μ*M*. The analysis time was 20 min per injection and the day-to-day precision was ±2–3%. The same method was also found to be useful in examining related conjugates, such as those based on the dye IRD40. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Deoxyadenosine triphosphate; Nucleotides

1. Introduction

Fluorescent labels are now commonly used in DNA sequencing [1] and in the detection of nucleotides that have been separated by capillary electrophoresis (CE) or high-performance liquid chromatography (HPLC) [2]. One group of labels that have been employed for this purpose are those based on near-infrared (near-IR) fluorescent dyes [3,4]. An example of such a label is IRD700 (see structure shown in Fig. 1). In water, this label has a maximum absorption of light at 685 nm, followed by the emission of fluorescence at near-IR wavelengths (emission maximum, 712 nm). One advantage of using this type of fluorescence for detection is that it occurs in a spectral region where there is relatively little absorption or emission due to other compounds that might be present in biological samples. This, plus the fact that IRD700 can be excited with

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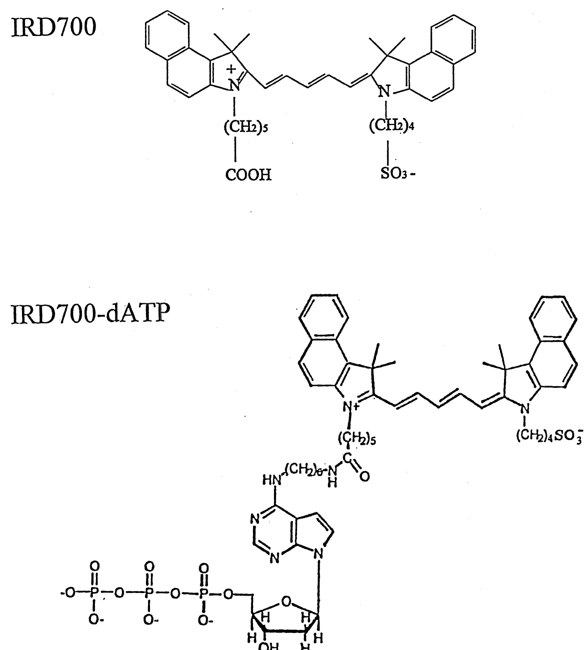

Fig. 1. Structures of IRD700 and IRD700–dATP.

commercially-available lasers, provides this approach with low background signals and limits of detection that extend into the attomole range [3].

In order to get good reproducibility when using these labels for DNA sequencing or related applications, it is necessary to ensure that IRD700-labeled nucleotides or similar conjugates contain the desired tag and represent a relatively pure, homogeneous preparation. Some examples of contaminants that might be present in these preparations include the original unconjugated dye or nucleotide, as well as hydrolysis products of the reactants or of the final labeled nucleotide conjugate. Any technique for monitoring these samples should ideally be able to discriminate between the final product and such contaminants. Furthermore, the ability to handle small amounts of sample and work at reasonably low concentrations is desirable because of the relatively high cost and small quantities of labeled conjugates that are normally available for testing.

This work will examine the development of a CE method for monitoring nucleotides labeled with IRD700 or related dyes [5]. This approach will first be examined by using the analysis of IRD700–dATP as an example. One reason that CE was chosen for this work is that all of the analytes or contaminants of interest are water-soluble compounds that have significant differences in their charge and/or size. CE was also appealing for use in this study because of its efficiency, relatively high speed and small sample volume requirements. In addition, by combining these properties with the use of a ligand that was added to the buffer (i.e., dTTP), this approach offers a possible means for discriminating between IRD700-labeled nucleotides and any unreacted dye. This report will discuss the optimum conditions for such an assay system and explore its use in the analysis of crude and purified samples of IRD700–dATP. The extension of this method to related dye conjugates will also be considered.

2. Experimental

2.1. Reagents

The deoxyadenosine 5′-triphosphate (dATP), deoxyadenosine 5′-diphosphate (dADP), deoxyadenosine 5′-monophosphate (dAMP) and deoxythymidine 5′-triphosphate (dTTP) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Mesityl oxide was obtained from Aldrich (Milwaukee, WI, USA). The IRD700-labeled dATP preparations, unconjugated IRD700, and IRD40 samples were provided by LI-COR (Lincoln, NE, USA). Other chemicals used in this study were of the purest grades available. All solutions were prepared with deionized water obtained from a Nanopure water system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

Experiments were performed using a BioFocus 3000 CE System from Bio-Rad (Hercules, CA, USA). The capillaries were 54 cm (50 cm effective length)×75 μm I.D. uncoated fused-silica columns from Polymicro Technologies (Phoenix, AZ, USA). Injections were performed in the vacuum injection mode of the BioFocus system, with the approximate sample volume being 6 nl. In the IRD700 work, the migration of solutes was monitored at both 254 and 680 nm, where the latter wavelength was used to

help identify those peaks which contained IRD700 or IRD700-labeled conjugates. In work with IRD40 samples, wavelengths of 254 and 772 nm (the absorbance maximum for IRD40) were used for detection. The applied voltage in the final optimized method was 25 kV (463 V/cm) and the standard running buffer was pH 9.5, 140 m*M* sodium borate [6]. The typical current under these conditions was 100 μA.

2.3. Methods

The samples were prepared in 1.5-ml microcentrifuge tubes by combining 100 μl of each test solution with 100 μl of running buffer, 798 μl of deionized water and 2 μl of mesityl oxide (an internal marker for electroosmotic flow). The standard samples of dATP, dADP and dAMP were prepared in the same manner but instead began with 1.0 m*M* stock solutions of each agent in deionized water; these stock solutions were then diluted with the pH 9.5 running buffer to give working standards with concentrations that ranged from 0.01 to 0.1 m*M*. The IRD700 working standards were similarly prepared to give final concentrations of 1.0–10.0 μ*M* by diluting a 20 μ*M* IRD700 stock solution that was made up in deionized water containing 6.5 m*M* triethylamine. All samples and standards were stored at 4°C until use.

All solutions used on the CE system were sonicated under vacuum for 15 min and filtered before use by passing them through MAGNA-R nylon 0.22-μm filters (MSI, Westboro, MA, USA). The CE capillary was conditioned prior to the sample analyses by rinsing it with a 0.1 *M* sodium hydroxide solution and equilibrating it overnight in this solution. The capillary was then washed with both deionized water and the pH 9.5, 140 m*M* borate running buffer. Each standard was injected in triplicate into the running buffer. Between injections, the capillary was washed with 0.1 *M* sodium hydroxide for 60 s, with deionized water for 60 s and with the running buffer for 30 s.

All studies were performed at 25°C.

3. Results and discussion

It has previously been shown that a pH 9.5, 140 m*M* borate buffer can successfully be used for the separation of ATP, ADP and AMP [6]. This same buffer was used in this study since dATP, dADP and dAMP were some of the possible contaminants in the IRD700–dATP samples. As shown in Fig. 2a, this buffer led to baseline resolution of these nucleotides

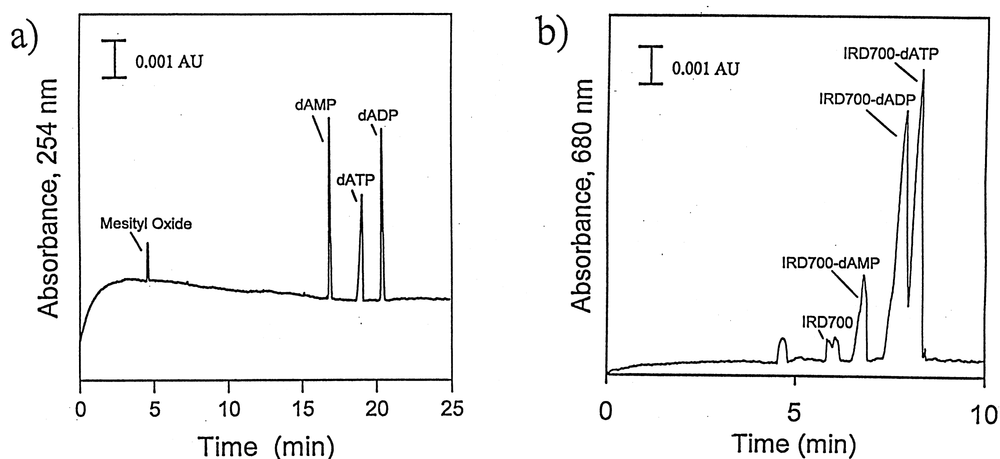

Fig. 2. Electropherograms obtained at 25 kV for the separation of (a) a mixture of dATP, dADP and dAMP and (b) a crude sample of an IRD700–dATP conjugate. The peak to the left in (a) is due to mesityl oxide, which was added to each sample as a marker for electroosmotic flow when working at 254 nm. The peak to the left in (b) is a solvent peak that appears at the same migration time as mesityl oxide; this is what was used as an internal migration marker for work at 680 nm. All other conditions are given in the text.

when using an applied voltage of 25 kV. The same conditions led to the production of several, well-defined peaks in the IRD700 conjugate samples (see Fig. 2b). Other running buffer salts, pH values and additives were considered in an attempt to further improve this separation (e.g., see Ref. [6] and the references cited therein), but no significant improvement in resolution was noted beyond the results that are shown in Fig. 2.

The operating voltage was another item that was considered when developing this method. This was done by varying the voltage from 10 to 25 kV while comparing the resulting separations of the unconjugated nucleotides and components found in the IRD700–dATP samples. Baseline resolution was seen between the peaks for dATP, dADP, dAMP over this entire range of applied potentials (e.g., see Fig. 2a). The peaks due to unconjugated IRD700 and a neutral marker were also baseline-resolved from all other peaks under these conditions (Fig. 2b). There was some overlap between two of the peaks that were believed to represent the conjugates of IRD700 with dAMP, dADP and dATP (i.e., see the last peaks on the right of Fig. 3), but this resolution was sufficient for quantitation and did not change appreciably in going from 10 to 25 kV. The use of higher voltages did not appear to greatly affect the band-broadening and degree of resolution seen between the peaks, but using these higher voltages did significantly decrease the overall run time. As a result, a voltage of 25 kV was selected for use throughout the remainder of this study.

The final selected conditions gave a separation in which all of the IRD700-related compounds were detected within 10 min and the unconjugated dAMP, dADP and dATP were detected within 20 min. The dATP, dADP, dAMP and unconjugated IRD700 peaks in the samples were identified by comparing their mobilities and migration times to those observed for standards containing these same compounds.

The conjugate peaks between IRD700 and dAMP, dADP or dATP were initially identified based on the appearance of three additional peaks in the crude IRD700–dATP samples that absorbed at 680 nm and that were not present in standards that contained only IRD700 or unconjugated nucleotides. To confirm that these peaks were adenosine nucleotides, $1.0 \cdot 10^{-4}$ *M* TTP was added to the running buffer. Since this is a complementary nucleotide to ATP, ADP and AMP, the binding of TTP to any solutes that contain these nucleotides should cause an apparent change in the size-to-mass ratios and migration times of these solutes when a sufficiently large concentration of TTP is present in the running buffer. This is illustrated in Fig. 4 for a mixture of dATP, dADP, and dAMP and for a crude sample of the IRD700–dATP conjugate. Peaks for all of the adenine-related nucleotides gave noticeable shifts in the presence of TTP, while the peak for the IRD700 did not. The

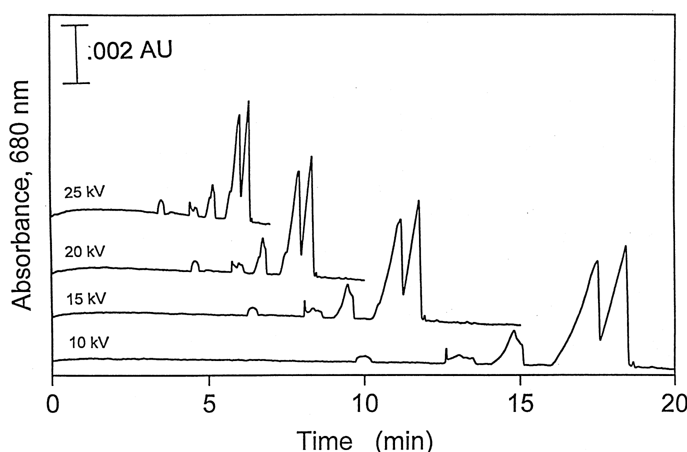

Fig. 3. Effect of applied voltage on the electropherograms obtained for a crude sample of an IRD700–dATP conjugate. The identities of the peaks are the same as given in Fig. 2b.

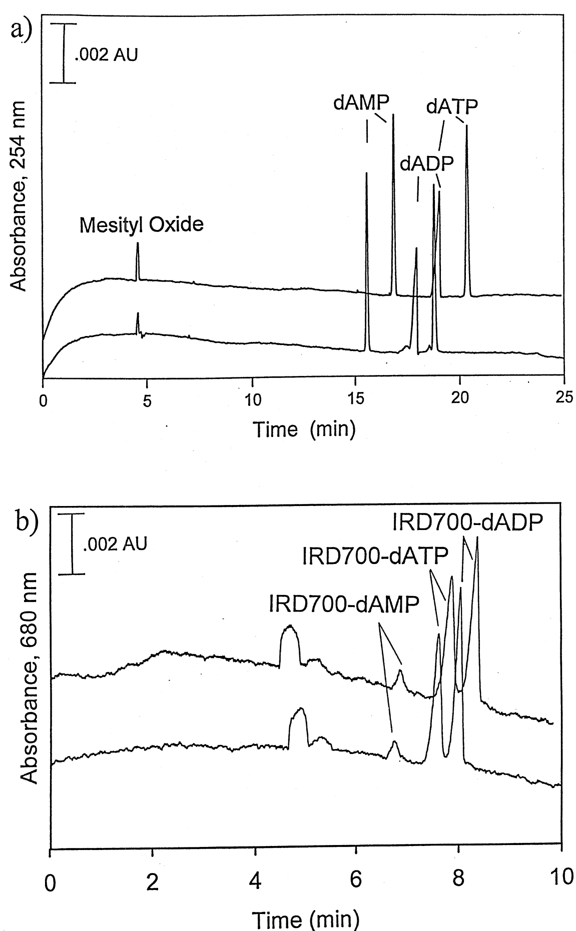

Fig. 4. Migration time shifts of (a) an adenosine nucleotide mixture or (b) a crude IRD700–dATP conjugate sample when dTTP (present in the lower runs) was used as an additive in the CE running buffer.

degree of this shift was significantly greater than the observed run-to-run variations in the peak mobilities that were measured, which had a typical precision of ±1.0% when compared to the observed mobility for a neutral marker in the same samples. This confirmed that the three new peaks seen in the crude IRD700 conjugate sample were indeed adenine-related nucleotides.

Calibration curves were generated by injecting several known concentrations of IRD700 and each unconjugated nucleotide. For the unconjugated IRD700, the lower limit of detection was found to be 1.0 μ*M* at a signal-to-noise ratio of three. This corresponded to a detection limit of $6 \cdot 10^{-15}$ mol for a 6-nl sample injection. The upper limit of detection extended up to at least 80 μ*M*; higher concentrations were not tested because of limited solubility of IRD700 in aqueous solutions. This solubility was improved by adding 6.5 m*M* triethylamine (TEA) to all samples during their preparation. A blank sample containing only the running buffer and TEA showed no noticeable absorption at either 254 nm or 680 nm, indicating that this additive did not interfere with the detection of IRD700 or any other sample components.

The linear range of the calibration curve for IRD700 extended from 1.0 to 80 μ*M*. The correlation coefficient for the best-fit line over this range was 0.9986 ($n=14$). This graph was then used to quantitate all peaks that were identified at 680 nm as containing IRD700 in their structure. The migration-corrected response factors that were used for this quantitation, as determined by an algorithm supplied with the BioFocus CE instrument, were 1.00 for IRD700, 1.14 for IRD700–dAMP, 1.30 for IRD700–dADP, and 1.36 for IRD700–dATP.

The detection of dATP at 254 nm gave a lower limit of detection of 1.0 μ*M* and a linear range that extend up to approximately 5.0 m*M*. The correlation coefficient for the best-fit line over this range was 0.9844 ($n=10$). The lower limits of detection for dADP and dAMP were also around 1.0 μ*M*, while the upper ends of the linear ranges were 1.0 m*M* and 2.5 m*M*, respectively. The correlation coefficients for dADP and dAMP over their linear ranges were 0.9988 ($n=9$) and 0.9997 ($n=10$).

The free dATP, dADP and dAMP showed no loss of peak area when samples containing these compounds were stored for up to three days at 4°C. The IRD700 solutions were stable for up to 18 h, but then began to show a slow decrease in peak area over time. The exact nature of this decrease is not known, but it is suspected that it is related to the relatively low solubility of IRD700 in aqueous solution.

The robustness of the CE method was investigated by making a series of injections over a period of several days. The day-to-day precision, as determined for a total of 12 injections over 3 days, was ±2.0–2.9% (1 RSD) based on peak areas. The within-day precision was similarly determined to be ±2.1–2.4%. The relative migration times for all of

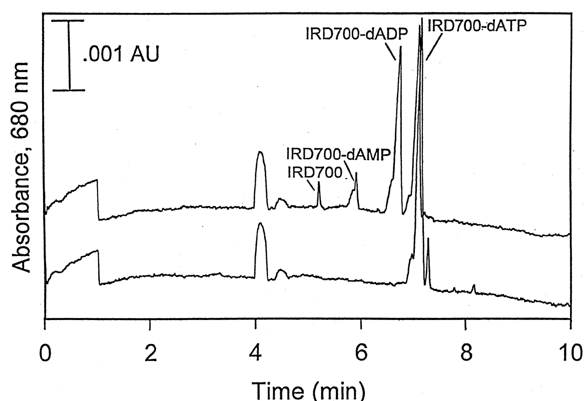

Fig. 5. Comparison of crude (top) and purified (bottom) IRD700–dATP conjugate samples.

the examined analytes had a day-to-day variation of approximately ±1.0–1.3% and a within-day precision of ±0.7–1.0%.

Fig. 5 shows an example in which this CE method was used to examine various preparations of IRD700–dATP. From this it can be clearly seen that there were significant differences in the crude and purified conjugate samples. With this CE method, it was possible to easily ascertain the relative amount of IRD700–dATP that was present in each sample along with the relative amount of impurities. For instance, the crude sample shown in the top of Fig. 5 gave an electropherogram in which the IRD700–dATP peak represented 45% of the total peak area and impurities made up 55% (not including the peak due to the marker for electroosmotic flow). But the purified sample in the bottom of Fig. 5 gave an electropherogram where 93% of the total area was due to IRD700–dATP and only 7% was due to other compounds. This information should be useful in the development of preparation and isolation methods for these conjugates and in the routine determination of conjugate purity.

The same method was found to be useful in the analysis of similar dye conjugates. An example is shown in Fig. 6, in which this technique was used for analyzing a purified sample of a dATP conjugate of IRD40, a near-infrared dye that is closely related to IRD700. The only change that was made in the method was the use of an alternative wavelength for detection of the dye conjugate. Baseline resolution between possible impurities and the desired conjugate was again obtained in an analysis time of less than 10 min for work performed at 772 nm. As was shown for IRD700, this technique allowed the relative purity of the conjugate to be estimated. In Fig. 6 this gave a result in which 93% of the dye was found

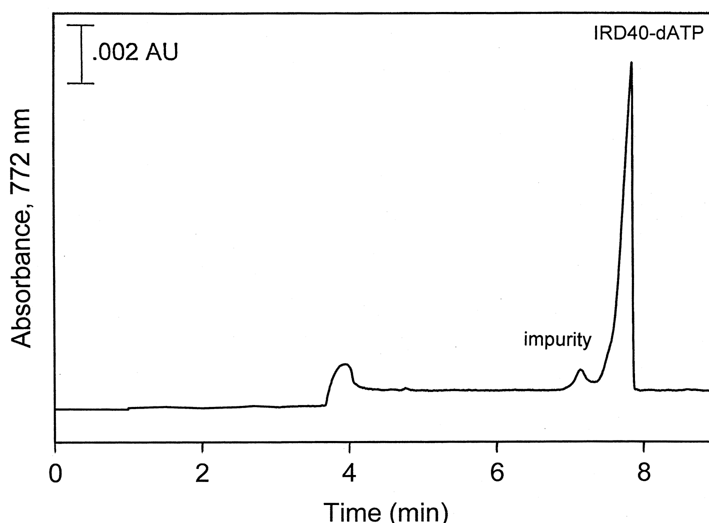

Fig. 6. Electropherograms for the analysis of an IRD40 conjugate. The peak to the left represents a solvent peak which comigrated with mesityl oxide; this peak is what was used as an internal migration marker for these samples.

to be incorporated into a dATP conjugate and the other 7% to be part of an impurity, believed to be IRD40–dADP.

4. Conclusion

In this study a method based on CE was developed to monitor dATP conjugates of the near-infrared fluorescent dye IRD700 and conjugates of the related dye, IRD40. It was possible with this approach to identify and quantitate IRD700–dATP along with several possible impurities, including the unconjugated forms of IRD700, dATP, dADP, and dAMP, as well as the conjugates of IRD700 with dADP and dAMP. Some advantages of this method include its small sample size requirement (6 nl of a 1:10 diluted sample) and its relatively short analysis time (20 min per injection for all contaminants or 10 min for only those that contain IRD700). These properties make this technique appealing for use in quality control monitoring of these conjugates. This method also offers a convenient way for assessing conjugate purity during the development and selection of preparation schemes for these chemicals.

Acknowledgements

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