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Selective Determination of Adenine-Containing Compounds by Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Capillary electrophoresis coupled with laser-induced fluorescence detection provides a selective analysis of mixtures of adenine-containing compounds (adenine, adenosine, cAMP, AMP, ADP, and ATP) that are derivatized using chloroacetaldehyde as a fluorogenic reagent. The components can be detected with linear response over the concentration range of 10^{-4} to 10^{-9} M, and the detection limit corresponds to 5 to 10 $\times$ 10^{-19} mol for each nucleotide. Analysis is achieved in less than 10 min with high separation efficiencies (up to 400,000 theoretical plates) and shows good reproducibility for migration times (0.5 to 1.0%) and peak heights (2.8 to 7.7%). To demonstrate the rapid analysis of small-volume samples, the adenine-containing compounds present in an oocyte from *Xenopus laevis* are determined.

The quantitative analysis of the purine and pyrimidine bases and their analogs is important in studies of their function in biological systems. Not only do these bases, nucleosides, and nucleotides (and modifications of these molecules) compose ribonucleic and deoxyribonucleic acids and coenzymes, but they are also involved in cellular development and proliferation (1), metabolism (2), energy storage and conversion (2), immune responses (3), and signal transduction (2). For example, ATP acts as a universal energy carrier in most organisms (2), cAMP behaves as a second messenger in the adenylate cyclase cascade (2), and guanine nucleotides are involved in visual excitation (2). The ubiquitous presence of these compounds in biological matter and the variety of roles played by them maintain a strong demand for improved sensitivity and accuracy in the analytical methods used in their investigation.

At present, high-performance liquid chromatography (HPLC) is the most widely used technique to analyze such biomolecules. Although uv absorbance detection is typically used with HPLC, it is plagued by interferences and its sensitivity is limited to the nanomole–picomole range. Fluorescence detection can also be used by reacting the compounds with a variety of derivatizing reagents (either pre- or postcolumn) (4–10). The limits of detection achieved with this approach are generally in the picomole–femtomole range. Nevertheless, with the recent trend in biology and biochemistry toward trace chemical analysis of ultrasmall-volume samples (11,12), the development of analytical technologies for subfemtomole (submicromolar concentration) detection of these biomolecules is warranted.

Because of its high separation efficiency, capillary electrophoresis (CE)[2] has also been used to analyze such compounds in recent years (3,13–15). The short pathlength characteristic of the capillary column, however, limits the method's sensitivity to the femtomole range when used with absorbance detection. As for HPLC, CE combined with this form of detection is prone to interferences, especially in the analysis of complex samples. Indirect fluorescence detection provides better mass limits of detection (<fmol), but the concentration detection limits remain poor (14). Using a large-frame Ar ion or a waveguide KrF laser for deep uv excitation, Milofsky and Yeung (15) used native fluorescence to detect certain nucleotide monophosphates and achieved high sensitivity ($\sim 10^{-7}$–10^{-8} M). This approach requires the use of either acidic or basic electrolytes for high fluorescence signal and thus restricts the choice of separation buffers. Consequently, a method is still needed that can

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[2] Abbreviations used: CE, capillary electrophoresis; LIF, laser-induced fluorescence; CAA, chloroacetaldehyde; ADE, adenine; ADO, adenosine; SDS, sodium dodecyl sulfate; RSD, relative standard deviation.

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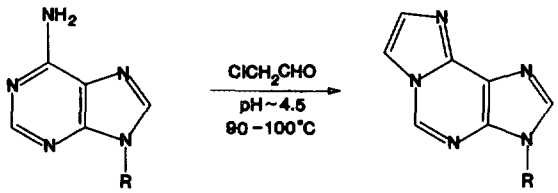

FIG. 1. The chloroacetaldehyde derivatization reaction that converts the adenine-containing compounds to fluorescent products.

separate and analyze these compounds with high sensitivity.

In this paper, we describe the use of laser-induced fluorescence (LIF) with capillary electrophoresis for the highly sensitive and selective analysis of adenine analogs. The adenine nucleosides and nucleotides are reacted with chloroacetaldehyde (CAA) to produce fluorescent products (as shown in Fig. 1) (5–8,16). These derivatives are separated with high efficiency by CE and detected on-column with a low-power He–Cd laser to achieve subattomole detection limits. Use of this methodology in the analysis of biological samples is also presented.

METHODS AND MATERIALS

Solutions. All solutions were prepared in water purified with a Milli-Q UV Plus (Millipore, So. San Francisco, CA) purification system. Phosphate buffers were made by dissolving either the monobasic or the dibasic form of sodium phosphate (J. T. Baker, Inc., Phillipsburg, NJ) at the appropriate concentrations. A 40 to 45% aqueous chloroacetaldehyde solution (Johnson Matthey Catalog Co., Ward Hill, MA) was diluted in 20 mM sodium phosphate buffer (pH 8.8) to produce the 0.15 M CAA (resulting pH 4.6) reagent used for derivatization reactions. Stock solutions of the analytes (2 mM) were made by dissolving adenine (ADE), adenosine (ADO), cAMP, AMP, ADP, and ATP (Sigma Chemical Co., St. Louis, MO), in 20 mM sodium phosphate buffer (pH 4.5). Subsequent nucleotide solutions at various concentrations were made from these stock solutions by diluting them with the same phosphate buffer. All solutions were filtered with 0.2-μm disposable syringe filters (Nalge Co., Rochester, NY), and the nucleotide stock solutions were refrigerated (at 4°C) when not in use.

Derivatization procedure. Equal volumes (20 μl) of the nucleotide and the CAA solutions were mixed in Eppendorf centrifuge tubes and capped. The solutions were then heated at 95 to 100°C for ~20 min using a multiblock heater (Lab-Line Instruments, Inc., Melrose Park, IL) to induce the reaction to occur and were cooled using an ice bath before being injected into the capillary.

Capillary electrophoresis apparatus. With the exception of the *Xenopus* oocyte work, all experiments were performed by using a CE–LIF apparatus that has a confocal detection design similar to that used previously for analysis of polycyclic aromatic hydrocarbons (17). The oocyte analyses were performed with a CE–LIF apparatus that uses a parabolic reflector (18). In both cases, a He–Cd laser (Liconix, Sunnyvale, CA) served as the excitation source (at 325 nm). The resulting emitted light was passed through a 375-nm longpass filter and a 400-nm bandpass (70 nm FWHM) filter. The photomultiplier tube voltage in both LIF instruments was 550 V. A positive high-voltage power supply (Glassman High Voltage, Inc., Whitehouse Station, NJ) provided the separation voltage. Fused silica capillaries (Polymicro Technologies, Inc., Phoenix, AZ) were flushed with sodium hydroxide (0.1 M) and water prior to use.

For comparison, CE runs were performed with uv absorbance detection (at 254 nm) on a P/ACE System 2000 (Beckman Instruments, Inc., Fullerton, CA).

Sample injection. Samples were injected by placing the capillary inlet in the sample solution at a height of 20.5 cm (11 cm for the oocyte study) above the outlet for 20 s. The transfer time between removal of the capillary from the sample vial and its replacement into the buffer reservoir was minimized to 1–2 s. The injection volume was determined using standard procedures (17); it measured ~0.5 nl for the 25-μm capillaries used with the confocal CE–LIF apparatus and ~3 nl for the 50-μm capillaries used with the parabolic reflector LIF apparatus.

Oocyte preparation. Xenopus laevis oocytes were stored in an egg buffer medium that consisted of 96 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 5 mM Hepes (adjusted to pH 7.6 with sodium hydroxide), 2.5 mM sodium pyruvate, 100 mg/ml penicillin, and 100 mg/ml streptomycin. Single *Xenopus* oocytes were transferred to Eppendorf tubes that contained 10 μl of the egg-storage buffer. An additional 20 μl of the storage medium and 30 μl of a solution that contained 1.5% SDS dissolved in 1:2 chloroform:methanol were added. The solution was vortexed after the egg was homogenized with the tip of a pipet. Thirty microliters of a solution of 1.5% SDS dissolved in chloroform was then added to yield two distinct layers. After the solution was centrifuged for 30 min at 10,000*g* using a microcentrifuge (Eppendorf, Hamburg, Germany), 20 μl of the top layer was withdrawn. The solvent from this layer was removed under vacuum for 45 min using a speed-vac apparatus before being reconstituted in 20 μl of the CAA solution for the derivatization reaction.

RESULTS AND DISCUSSION

Reaction conditions. The derivatization reaction was carried out at several different pH values, and the optimum pH value (highest fluorescence generated from products) was found to be ~4.5. This value agrees with

that of previous work (5). The CAA concentration used in the reactions was a factor of 100–1000 times higher than the total analyte concentration, as suggested by Sonoki *et al.* (5).

The effects of the reaction temperature and the reaction time on the derivatization efficiency were also studied, though not in detail. The best results were obtained when the reaction was carried out at 90–100°C for ~20 min. Below this temperature range the reaction did not go to completion for most of the analytes, and significant breakdown of products occurred above this range. Similarly, the reaction time of 20 min was chosen as a compromise between the point at which there were sufficient derivatization products and the point at which significant decomposition of the products would occur.

Separation conditions. The pH of the 20 mM phosphate buffer used to separate the samples did not significantly affect the relative migration times of the analytes. A pH of 8.8 worked well and represented a balance between the resolution of the separated components and the total time required for separation. Although the addition of 1.5% SDS improved the separation between ADE and ADO, increased background fluorescence was also observed, possibly because of contaminants in the SDS. Hence, we decided to discontinue the use of SDS for routine analyses performed in this study. (The use of SDS may be useful in analyzing complex biological samples because of its ability to deactivate enzymes that might otherwise degrade the nucleotides being studied.) The short exposure of the derivatized components to the phosphate buffer (pH 8.8) during separation did not significantly affect the fluorescence intensities.

Figure 2 shows an electropherogram for a typical CE separation of a sample that contains approximately 2 μM each of the six adenine compounds. Separation efficiencies of up to 400,000 theoretical plates are achieved and all of the nucleotide peaks are baseline resolved. (The adenine and adenosine peaks are fully resolved only when SDS is added to the buffer.) Flushing the column with the separation buffer between runs was essential for maintaining the quality of the separations.

Reproducibility and linearity of measurements. Reactions of solutions that contained the same concentrations of the adenine nucleotides and the CAA reagent were carried out in four separate vials to test for reproducibility. All four vials were placed simultaneously in the multiblock heater to minimize differences in reaction conditions. The relative standard deviations (RSDs) for the six analytes ranged from 0.5–1.0% for the migration times, 2.8–7.7% for the peak heights, and 4.4–12.4% for the peak areas. As is typical, the RSD values increased for analyte peaks that eluted at later times. Automating the sample-injection procedure or minimizing the difference in pH between the reaction and separation buffers may lower RSD values in the future. Nevertheless, the reproducibilities exhibited in the present study show that quantitative analysis of adenine-containing nucleotides is feasible.

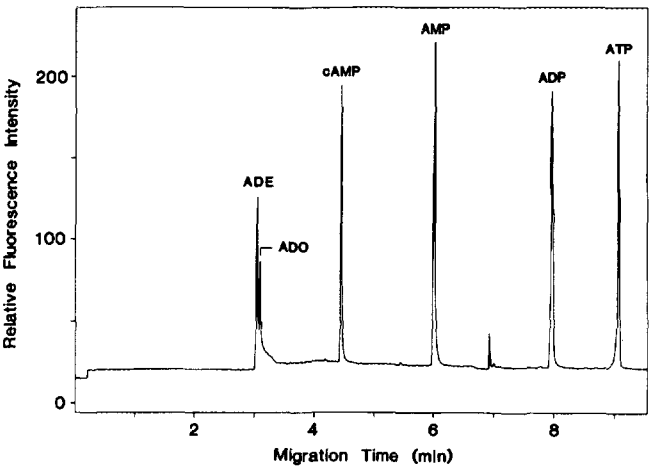

FIG. 2. Electropherogram of approximately 2 μM each of adenine (ADE), adenosine (ADO), cAMP, AMP, ADP, and ATP derivatized with CAA and analyzed with the confocal LIF apparatus. The peak between AMP and ADP is an unknown impurity peak. The column dimensions were 25 μm i.d. and 60 cm in length (40 cm to detector). 20 mM sodium phosphate was used as the separation buffer at an applied separation voltage of 20 kV. Other details are given in the text.

Despite some decomposition of the fluorescent products during the reaction, linear calibration curves for both peak heights and peak areas (vs concentration) were obtained. Reactions of different concentrations of the nucleotides were carried out simultaneously and then analyzed by CE. Plots of both the peak heights and peak areas versus concentration of the nucleotides (cAMP, AMP, ADP, ATP) were linear, and correlation coefficients were >0.980 for concentrations between 10^{-9} and $\sim 10^{-4}$ M. Detection of adenine and adenosine at concentrations below 10^{-6} M was difficult because of interfering (unknown) peaks that coeluted with these two compounds.

Limits of detection. The limits of detection for the nucleotides derivatized with CAA and analyzed with CE–LIF (confocal design) ranged from 5 to 10×10^{-19} mol ($1–5 \times 10^{-9}$ M), a factor of 1000 to 10,000 lower than limits obtained using absorbance detection (experiments performed on the Beckman P/ACE system). Because of interference problems, the detection limits for ADE and ADO were 100 to 1000 times poorer than for the nucleotides. The sensitivity can potentially be improved by using a He–Cd laser with more power (the analyte molecules were not significantly photobleached at the 5–10 mW range of laser power used in this study) or by closer matching of the laser excitation wavelength (325 nm in this case) to the excitation maximum (~290 nm) for the derivatized adenine-containing compounds.

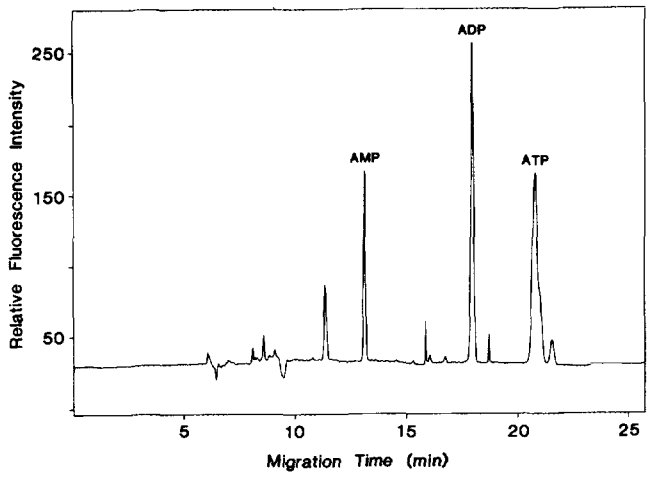

FIG. 3. Electropherogram of a sample containing the contents of a single *Xenopus* oocyte derivatized with CAA (using the parabolic reflector LIF apparatus). The column used was 50 μm i.d. and 90 cm in length (45 cm to detector). Other details are given in the text.

Possible applications. To demonstrate application of this procedure to the analysis of a biological sample, we chose to study adenine nucleotides from *X. laevis* oocytes. Contents of a single oocyte were derivatized before they were injected into the CE column. The resulting electropherogram (no added standards) is shown in Fig. 3. The nucleotide peaks are easily visible (peaks were identified by spiking with standards), and few interfering peaks appear. (The asymmetry in the ATP peak may be because of an interfering compound coeluting with ATP under the buffer conditions used in the separation.) Thus, the derivatization procedure appears to be highly selective for adenine-containing compounds. Although it has been reported that CAA can also derivatize cytidine-containing nucleotides (16), the conditions used here do not seem to favor such reactions.

The sensitivities achievable with this procedure and the small-volume capabilities of CE suggest that this combined CE–LIF technique should be useful in biological studies that involve limited sample volumes. An example is the development of powerful techniques for analyzing cellular contents at the single-cell level for the purpose of elucidating mechanisms of various biological processes (11,12). Although the oocyte study represents preliminary work, the practicability and potential have been demonstrated for studying adenine-containing nucleotides in biological samples by CE–LIF using chloroacetaldehyde as a derivatizing agent.

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