Journal of Chromatography, 608 (1992) 171–174
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 435

Capillary electrophoresis of fluorescein–ethylenediamine–5′-deoxynucleotides

Wenni Li*, Adel Moussa** and Roger W. Giese

Department of Pharmaceutical Sciences in the College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (USA)

ABSTRACT

Fluorescein–ethylenediamine conjugates of the four major deoxynucleoside-5′-monophosphates were subjected to capillary electrophoresis with laser fluorescence detection. Control of pH was an important parameter controlling resolution, as anticipated. For example, the four conjugates were resolved at pH 10.4, but negligibly at pH 8.7. Apparently secondary effects on solute migration such as conformation or solvation play a role, since the larger conjugate of adenine migrated faster than that of cytosine at pH 10.4, where each has the same charge. Further, the guanine and thymine conjugates, in spite of their different size, co-migrated at pH 11.5. The method promises to be useful for the detection of DNA adducts.

INTRODUCTION

Previously we reported the separation of fluorescein-labeled deoxynucleotides by high-performance liquid chromatography (HPLC) [1]. The purpose of achieving the separation is to facilitate the detection of covalent damage to DNA, termed DNA adducts, by environmental or endogenous chemicals. The overall analytical scheme that we are pursuing is to sequentially purify the DNA from a biological sample, isolate the DNA adducts as damaged deoxynucleotides, label the latter with fluorescein, and then achieve sensitive detection of the resulting fluorescein-labeled deoxynucleotide DNA adducts. This basic strategy is being pursued also by others [2,3]. Potentially the measurement of DNA adducts can help define how much of the human burden of cancer and genetic disease results from exposure to toxic chemicals and radiation.

Here we extend our prior HPLC work by examining the separation of these types of compounds by capillary electrophoresis (CE). Because of its speed, resolution, ease of column cleaning, formation of sharp peaks to enhance detection, and tiny injection volumes to conserve precious samples, capillary electrophoresis should be useful for the detection of DNA adducts.

EXPERIMENTAL

Chemicals and reagents

Trizma base [tris(hydroxymethyl)aminomethane, or Tris] was purchased from Sigma (St. Louis, MO, USA). Boric acid, HPLC-grade acetonitrile and 0.22-μm MSI Cameo filters were from Fisher Scientific (Bedford, MA, USA). The fluorescein–ethylenediamine–deoxynucleoside-5′-phosphates (F-EDdNMPs) were prepared as described [1] using Isomer II FITC (fluorescein-6-isothiocyanate) from Molecular Probes (Eugene, OR, USA). All solution compositions were v/v unless indicated otherwise.

Correspondence to: Dr. R. W. Giese, Mugar Building, Room 122, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA.

* Present address: American Cyanamide Co., Agriculture Research Division, Princeton, NJ 08540-0400, USA.

** Present address: Covalent Associates, Inc., Woburn, MA 01801, USA.

0021-9673/92/$05.00

Capillary electrophoresis with laser fluorescence detection

A laboratory-built apparatus was used for CE. The laser-fluorescence detector was designed and built by Dr. Edward S. Yeung at Iowa State University. It utilized an argon ion laser (Model 2211-30SL; Cyonics, CA, USA) to give excitation at 488 nm. A Model PS/EH60R01.5 regulated high-voltage d.c. power supply (Glassman High Voltage, NJ, USA) was used. CE was performed in a 95-cm long fused-silica capillary (75 μm I.D.; PolyMicro Technologies, Phoenix, AZ, USA). The polyimide coating of the capillary was burned off to form a flow cell 50 cm from the injection end (grounded anode end) of the capillary. All separations were performed at 15 kV and samples were injected hydrodynamically: anode end 5 cm higher for 20 s.

New capillary columns were cleaned initially by syringe-filling (until 4 drops emerged, which was done for all syringe steps) with methanol–water (1:1), followed by 0.1 *M* NaOH, with standing for 30 min each. After washing with water and then buffer, the buffer of interest was then subjected to 30 min of electrophoresis followed by standing overnight. Periodically (about once per week) the capillary was cleaned by syringe injection of 0.1 *M* NaOH, standing for 30 min, similarly injecting buffer, and then operating the electrophoresis for 1 h before samples were injected.

Solutions

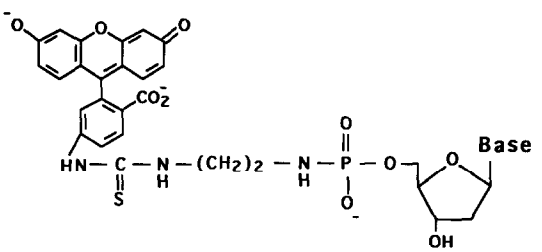
pH 8.7 Buffer. This was prepared by combining 0.8 ml of stock buffer (0.5 *M* Tris, 0.5 *M* boric acid, stored at room temperature), 35 ml of water, and 4 ml of acetonitrile, giving pH 8.7. This buffer (10 m*M* Tris–borate, 10% acetonitrile) was filtered (0.22 μm) and degassed (bubbling with helium for 15 min) prior to use as a diluent to prepare solutions for injection. The other buffers were prepared by titrating the 10 m*M*, pH 8.7 solution to the desired pH with 2 *M* NaOH.

F-ED-dNMP Stock

The stock solution of each F-ED-dNMP was obtained as an HPLC peak from a C_{18}-silica column in a mobile phase of 5 m*M* acetic acid with an acetonitrile gradient. Immediately after collection, the pH was adjusted to 8.7 by the addition of 0.5 *M* Tris–borate. This solution was kept in a polypropylene tube, and all dilutions, unless indicated otherwise, were made in polypropylene tubes, and Fisher Brand 1.5-ml polypropylene snap cap tubes. All dilutions were made using polypropylene tips. The stock solutions were stored in the dark at −20°C.

RESULTS AND DISCUSSION

The four common deoxynucleotides (dCyd-5′-P, dAdo-5′-P, Thd-5′-P and dGuo-5′-P) were first conjugated to ethylenediamine via the phosphate moiety, and then reacted with fluorescein-isothiocyanate. The resulting F-ED-dNMPs were purified by HPLC, and then subjected, both individually and as a mixture, to capillary electrophoresis with laser fluorescence detection. Shown here is a generalized structure of our analytes.



The first pH that we investigated for the electrophoretic separation was 8.7. The fluorescence of fluorescein reaches a maximum around pH 8.0; pH 8.6 has been recommended for the detection of fluorescein conjugates [4]. A Tris–borate buffer was selected primarily because of the double dose of buffering available from the pK_a values of 8.3 for Tris and 9.2 for borate (25°C). Thus the alkaline pH of 8.7 is half-way between these two pK_a values and close to the recommended pH of 8.6. We included 10% acetonitrile in the buffer since fluorescein, as a prelimary test solute, gave a narrower peak when this was done (data not shown).

At pH 8.7 the four F-ED-dNMPs essentially coelute by capillary electrophoresis, as shown in Fig. 1A. This observation is not surprising since the effective mobilities of uridine, cytidine and adenosine 5′-monophosphates were found to be very similar previously in this pH zone by isotachophoresis [5]. Further, the extra structural bulk contributed by the fluorescein-ethylenediamine moiety in our compounds probably reduces the degree to which the

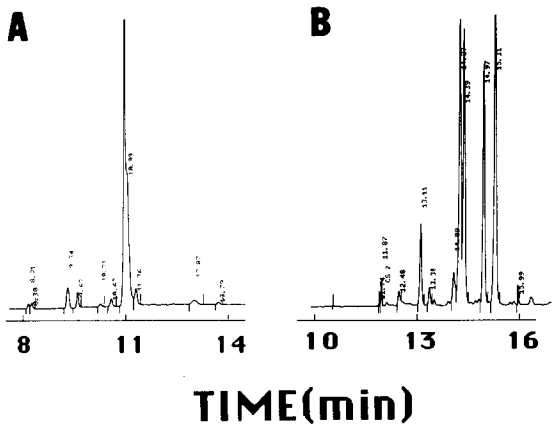


Fig. 1. Electropherograms of a mixture of fluorescein–ethylenediamine conjugates of dCyd-5′-P, dAdo-5′-P, Thd-5′-P and dGuo-5′-P at pH 8.7 (A) and 10.4 (B). The elution order of the compounds in (B) (4 major peaks) is C > A > T > G, the opposite order of their electrophoretic mobilities. In (B), the impurity at 13.11 min (contributed by the F-ED-dGMP sample), and the other background peaks, are unknowns.

structural differences among the four bases influence the frictional profiles of the compounds. Further, at pH 8.7 the four bases are essentially uncharged, since the nearest pK_a for any of them is the one at 9.7 for dGua-5′-P [6]. Next is that at 10.0 for Thy-5′-P [6]. No acidic pK_a values have been reported for the bases in dAdo-5′-P and dCyd-5′-P. The constant effective mobilities of cytidine- and adenosine-5′-monophosphate from pH 9 to 12 [5] indicates that neither has an acidic pK_a in or near this range.

Mononucleotides can be resolved by capillary electrophoresis under mildly acidic conditions [7–10], largely due to pK_a differences among the bases in the pH range of 2–4 [5]. A separation of some of them was also reported near neutral pH [11]. However, as pointed out above, we need to detect our compounds at an alkaline pH to maintain the intense fluorescence of fluorescein. Thus we next tested pH values above 8.7 for the running buffer.

As shown in Fig. 1B, all four compounds can be resolved at pH 10.4. The elution order fits the pK_a values, to the extent they are known (see above). However, it is not clear how F-ED-dAMP achieves a higher mobility than F-ED-dCMP, since neither base is ionized at this pH, and adenine is larger than cytosine. Secondary effects on mobility such as conformation or solvation apparently are playing a role. Perhaps the fact that the calculated dipole moments for cytosine and adenine are 7.6 and 2.9, respectively [12], is relevant.

The resolution degrades at pH values above 10.4 (data not shown). For example, at pH 11.5, the highest pH tested, essentially two peaks are observed when a sample containing the four compounds is injected: one at 12.2 min for the cytosine and adenine conjugates, and one at 14.4 min for those of guanine and thymine. The latter co-migration is another surprise, given the different sizes of the guanine and thymine bases, and their active role in the electrophoretic migration of the compounds at this pH, where each base is fully ionized.

Greater understanding is needed of the factors which determine the separation, or lack thereof, of small molecules possessing the same charge and type of charge in electrophoresis. Clearly small size differences can be important, *e.g.* the resolution of alkyl sulfates up to C_{12} by CE [13]. However, as an example that is more similar to one of our observations, Nielsen *et al.* [14] observed three peptides (5-, 6- and 8-mer) to co-elute at a pH where each possesses the same charge.

CONCLUSIONS

CE promises to be useful for the detection of fluorescein-labeled DNA adducts, particularly because of the broad pH range that it can utilize to take advantage of distinctive pK_a values that many adducts possess [6,15].

Given the marginal differences in electrophoretic mobilities for the compounds tested under certain conditions, we plan to investigate more complex conditions involving secondary retention mechanisms for more control over resolution.

CE has certainly rejuvenated interest in the electrophoretic separation of small molecules. This calls for further study of the factors which determine the frictional profiles of such compounds.

ACKNOWLEDGEMENT

This work was funded by the Office of Health and Environmental Research Division, Department of Energy, as Grant DE-FG02-90ER60964. Publication number 541 from the Barnett Institute.

REFERENCES

1 A. N. Al-Deen, D. C. Cecchini, S. Abdel-Baky, N. M. Abdel Moneam and R. W. Giese, *J. Chromatogr.*, 512 (1990) 409.
2 M. Sharma, R. Jain and T. V. Isac, *Bioconjugate Chem.*, 2 (1991) 403.
3 O. J. Kelman, K. T. Lilga and M. Sharma, *Chem.-Biol. Interactions*, 66 (1988) 85.
4 R. C. Nairn, *Fluorescent Protein Tracing*, Williams & Wilkins, Baltimore, MD, 1969.
5 T. Hirokawa, S. Kobayashi and Y. Kiso, *J. Chromatogr.*, 318 (1985) 195.
6 G. D. Fasman (Editor), *Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, FL, 3rd ed., 1975.
7 W. G. Kuhr and E. S. Yeung, *Anal. Chem.*, 60 (1988) 2642.
8 L. Gross and E. S. Yeung, *J. Chromatogr.*, 480 (1989) 169.
9 R. Takigiku and R. E. Schneider, *J. Chromatogr.*, 559 (1991) 247.
10 V. Dolnik, J. Liu, J. F. Banks and M. V. Novotny, *J. Chromatogr.*, 480 (1989) 321.
11 T. Tsuda, K. Takagi, T. Watanabe and T. Satake, *J. High Resolut. Chromatogr., Chromatogr. Commun.*, 11 (1988) 721.
12 W. Saenger, *Principles of Nucleic Acid Structure*, Springer, New York, 1984, p. 107.
13 M. W. F. Nielen, *J. Chromatogr.*, 588 (1991) 321.
14 R. G. Nielsen, R. M. Riggin and E. C. Rickard, *J. Chromatogr.*, 480 (1989) 393.
15 B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, 1983.