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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 945–950

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# Selective fluorogenic derivatization of a peptide nucleic acid trimer with naphthalene-2,3-dicarboxaldehyde

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Received 22 March 1996; accepted 16 July 1996

**Abstract**

The reversed-phase high-performance liquid chromatography of a Peptide Nucleic Acid (PNA) trimer has been studied after its preseparation fluorogenic derivatization with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide (NDA/CN). Trace levels of the PNA trimer were determined in cell homogenate samples containing the PNA trimer at prederivatization concentrations as low as 48.9 ng $ml^{-1}$. The sample pretreatment operations included a deproteination step, achieved by ultra-filtration, followed by fluorogenic derivatization (NDA/CN). Subsequently, to achieve adequate selectivity, the fluorescently labeled PNA was subjected to high performance anion exchange chromatography prior to quantitation via fluorescence detection. The various problems encountered during sample pretreatment and separation of derivatized PNA trimer in biological samples are presented and discussed. © 1997 Elsevier Science B.V.

*Keywords:* Peptide nucleic acids; NDA/CN

## 1. Introduction

There has recently been much interest in the possibility of using peptide nucleic acids (PNAs, Fig. 1) to control gene expression in mammalian cells. PNAs are analogues of oligonucleotides that contain peptide bonds substituted for the normal sugar-phosphate ester polymer [1]. These molecules are capable of targeting either DNA or RNA inside a cell to inhibit gene expression and additionally may block extracellular protein production [2]. PNAs have been shown to have several unique properties: (i) binding to the complementary DNA or RNA with high affinity; (ii) resistance to enzymatic degradation by DNase or RNase; and (iii) relative ease and economy of synthesis on a large scale. These and other properties often make PNAs ideal as antisense agents in the treatment of cancer and viral diseases [3].

The trace analysis of PNAs, especially in biological samples, constitutes a significant analytical challenge as low concentrations have to be determined against a potentially high background of interfering compounds. In the present study, a PNA trimer was derivatized with naphthalene-2,3-

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*PII* S0731-7085(96)01927-9

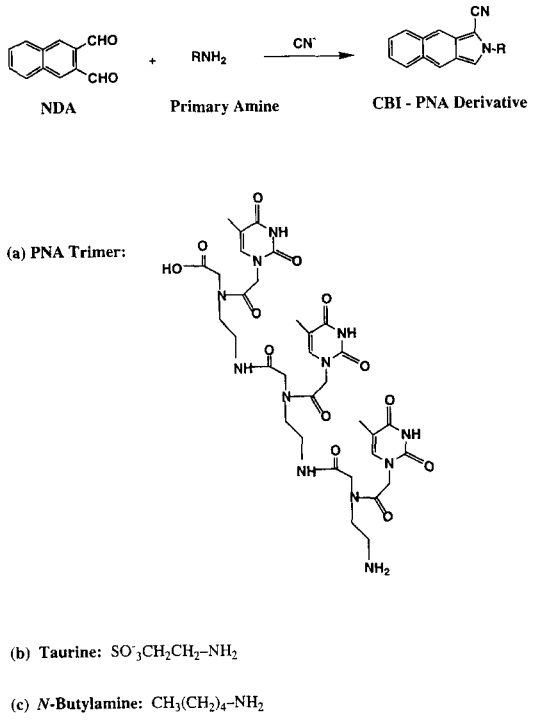


Fig. 1. Fluorogenic reaction of (a) the PNA trimer, (b) taurine, (c) *N*-butylamine with NDA in the presence of cyanide ion to produce *N*-substituted CBI derivatives.

dicarboxaldehyde (NDA) in the presence of cyanide (NDA/CN) and quantitated using conventional fluorescence detection. This reagent system reacts with primary amine groups to form efficiently fluorescent *N*-substituted 1-cyanobenz[f]isoindole (CBI) derivatives [4] and, as would be expected, was shown in the present study to readily label PNAs.

## 2. Experimental

### 2.1. Apparatus

All LC determinations were performed using a modular system consisting of a Shimadzu (Baltimore, MD, USA) Model SCL-6A system controller, Model LC-6A pumps, Model RF-535 fluorimetric detector (xenon lamp, excitation $\lambda =$ 440 nm, emission $\lambda = 490$ nm), and a Perkin-Elmer (Norwalk, CT, USA) Model ISS-100 autoinjector. The data were collected using a Perkin- Elmer Model LC-100 chart recorder. Reversed-phase [ODS Hypersil, 5 μm, 250 × 4.6 mm; Alltech (Deerfield, IL, USA)] and ion-exchange [PRP-X100, 250 × 4.1 mm; Hamilton (Reno, NV, USA)] LC columns were used. The Centrifree® micropartition system was purchased from Amicon (Beverly, MA, USA). All chromatographic separations were conducted at ambient temperature with a volumetric flow rate of 1.0 ml $min^{-1}$ unless stated otherwise.

### 2.2. Chemicals and reagents

The PNA trimer was synthesized according to the procedure of Egholm et al. [1,2]. NDA was synthesized as per the previously described procedure [5]. Acetonitrile (HPLC grade), methanol (HPLC grade), sodium hydroxide, sodium phosphate, citric acid and ammonium formate were obtained from Fisher Scientific (St. Louis, MO, USA). Sodium cyanide and taurine were from Sigma (St. Louis, MO, USA) and *N*-butylamine was purchased from Matheson Coleman and Bell (Norwood, OH, USA). *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate (Hepes) and Hanks' balanced salt solution (HBSS) were obtained from JRH Biosciences (Lenexa, KS, USA). Distilled, deionised water was used throughout the investigation.

### 2.3. Stock solutions

Solutions of NDA (1.0 mM) were prepared in methanol and used for a maximum of 1 week [6] (stored at 4°C protected from light). Aqueous solutions of NaCN (10 mM), *N*-butylamine (100 mM), sodium phosphate buffer (pH 2.6, 200 mM) and sodium borate buffer (pH 10.6, 100 mM) were prepared as required. Aqueous standards of PNA trimer were prepared in HBSS buffer, pH 7.4 and stored at −70°C when not in use.

### 2.4. Derivatization procedures

PNA trimer was derivatized in borate buffer (pH 10.6) by mixing the following solutions in the stated order to give a final volume of 250 μl:

borate buffer (100 μl, 100 mM); PNA trimer (100 μl, variable concentrations); NaCN (25 μl, 10 mM); and NDA (25 μl, 1.0 mM). After 10 min, the productive derivatization was complete and the excess NDA was chemically scavenged (5 additional min reaction time) by the addition of *N*-butylamine (100 μl, 100 mM). The *N*-butylamine scavenged product was selectively removed by liquid-liquid extraction using ethyl acetate (500 μl). The remaining aqueous fraction was transferred (200–250 μl) to a glass vial and stored at room temperature protected from light pending LC determination.

### 2.5. LC methodology

The LC mobile phases were prepared by filtering the appropriate buffer solution through a 0.45 μm Nylon 66 membrane filter (Microns Separations Inc., Westboro, MA, USA) and degassing prior to use. The derivatized PNA samples were subjected to reversed-phase LC using the following step gradient conditions and parameters: solvent A, phosphate buffer (pH 2.6, 0.2 M); solvent B, acetonitrile; gradient parameters, 0–15 min, 30% B, 15–25 min 60% B, 25–36 min 30% B.

Cell homogenate working standards containing PNA trimer were prepared by the addition of variable concentrations of stock solutions of PNA trimer to Caco-2 cell suspensions. Immediately after preparation, these standards were subjected to the following procedure. Borate buffer (1.0 ml) was added to a cell suspension containing the PNA trimer. The cells were sonicated using an ultra-sonicator for 2 min (Model W-385, Heat Systems Ultrasonics Inc. (Farmingdale, NY, USA). Ice cold acetonitrile (1.0 ml) was added to the cell homogenate, which was then subjected to centrifugation (4300 × *g*, 5000 rpm, 15 min). The supernatant was collected, evaporated to dryness, resuspended in 1.0 ml HBSS buffer and then subjected to ultra-filter centrifugation (3000 MW cut-off) using the Amicon Centrifree® micropartition system. The ultrafiltrate was collected and a 100 μl aliquot derivatized according to the previously described procedure. The derivatized sample was analyzed via LC using the anion exchange column operated according to the following conditions and parameters: solvent A, ammonium formate buffer, 0.15 N, pH 5.5; solvent B, acetonitrile; gradient conditions, 0–10 min 35% B, 10–35 min 70% B, 35–50 min 35% B.

## 3. Results and discussion

### 3.1. Derivatization

Chemical derivatization for the enhancement of detectability was required in the present investigation. Precolumn rather than postcolumn derivatization was selected after consideration of several fundamental aspects concerning the implementation of an analytical derivatization reaction into the overall analytical method. In particular, the high flexibility of reaction solvent composition and reaction kinetics that are generally available in precolumn derivatizaton were considered attractive, along with the obvious need to perform an efficient separation with minimal mobile phase restrictions. Unfortunately, when using postcolumn derivatizations one often compromises separation conditions to accommodate reaction optimization. Moreover, it was initially anticipated that reversed-phase liquid chromatography of the native analyte, a highly polar species, would provide little chromatographic selectivity as compared to the derivatized product. Therefore, based on these considerations, precolumn fluorogenic derivatization with NDA/CN was selected as the preferred approach at the onset of method development.

The reaction of NDA/CN with the PNA trimer is shown in Fig. 1. The two component reagent functions by reaction of one of the NDA aldehyde groups with the analyte primary amine to form a Schiff base, followed by addition of cyanide to form a secondary amine. This intermediate subsequently undergoes cyclization with the remaining aldehyde followed by elimination of water to complete formation of the CBI ring system [4,7]. Based on the results of earlier pH-rate optimization studies [8], the derivatization was performed at pH 10.0. Quenching of the derivatization reaction was accomplished through the use of *N*-butylamine, which allows the preferential liquid-liquid

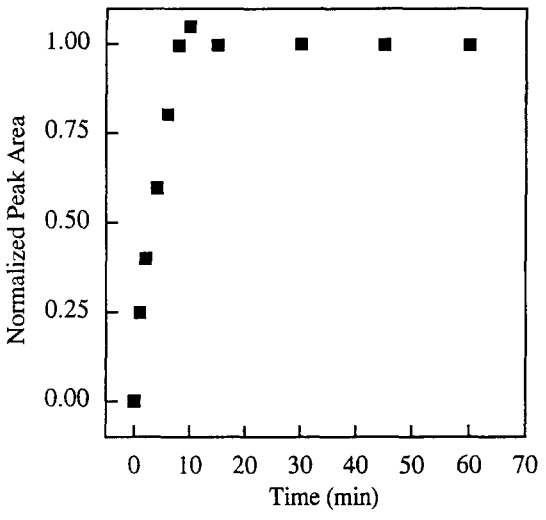


Fig. 2. Peak area as a function of time for the NDA/CN derivatization of the PNA trimer. Derivatization conditions were as described in the Experimental Section. Each point represents the average of three determinations. Error bars are within each point.

extraction of excess NDA through the formation of a nonpolar product. *N*-butylamine was chosen over taurine, which is often the quenching reagent of choice for NDA derivatizations [9]. Taurine usually allows the selective separation of excess NDA from analytes through the formation of a highly polar product. This causes the chemically transformed excess NDA to elute in the void volume during reverse phase LC [10]. Unfortunately due to its polar nature, the CBI-derivative of the PNA co-eluted with the CBI-taurine derivative. The derivatization reaction time for all samples was determined to be 10 min based on the yield time-curve shown in Fig. 2 for the derivatization of PNA trimer. After this time, maximal peak area was observed, with the derivative remaining stable (95% area maintained as ascertained by subsequent LC determinations) for a period of at least 24 h when the reaction mixture was kept at ambient temperature. During this period, no additional peaks that could be attributed to product degradation were observed.

The linearity of the assay and limit of quantitation of the derivatized product in water were investigated using reversed-phase LC. Each calibration point was generated by averaging the results of three determinations. The resulting data were subjected to linear regression analysis, the results of which are described by the following equation and parameters: CBI-PNA (μM) = 0.099 (μM/cm) [PH (cm)] − 0.003 (μM), $r = 1.0$. The mass limit of detection (MLOD) (injection volume = 100 μl) was determined to be 1.38 ng (pre-derivatization concentration 13.87 ng $ml^{-1}$). Fig. 3 shows sample chromatograms obtained during the generation of calibration plot and limit of quantitation data. No significant variations in retention time were noted using the ODS Hypersil column over a period of 2 months with approximately 400 injections, despite the use of a pH 2.6 mobile phase.

### 3.2. Cell homogenate samples

Different sample preparation techniques were evaluated for the preseparation isolation of the PNA trimer from other cellular proteins. After screening several approaches, acetonitrile-mediated protein precipitation followed by ultrafiltration was selected as the method of choice. Other standard techniques, such as solid phase extraction (including C8, C18 and strong anion exchange sorbents) or liquid-liquid extraction did not effectively separate the PNA trimer from interfering proteins, probably due to similarity in charge and dipole characteristics. The precipitation/ultrafiltration approach resulted in recoveries of about 75 (± 5.2)%, $n = 3$. This reproducible but non-quantitative recovery was smaller than desired, perhaps due to protein binding, inclusion of analyte by cell constituents and/or the result of diminishing cross membrane flux during the ultrafiltration step due to the high protein content of cell samples. The addition of an equal volume of water to the cell homogenate samples improved the recovery but the resulting sample dilution negatively effected the sensitivity of the analysis. This two-step sample pretreatment procedure eliminated most of the large interfering proteins from cell homogenate samples allowing for subsequent LC analysis.

### 3.3. HPLC assay for cell homogenate samples

A Hamilton PRP-X100 (quaternary ammonium functionalized crosslinked polystyrene) LC column was used for analysis of cell homogenate samples. This support afforded the required chromatographic resolution between the CBI-PNA derivative and the cellular matrix components not removed during sample preparation (reversed-phase LC using an ODS Hypersil packing was initially attempted but it did not provide the necessary selectivity). The polymeric ion-exchanger has several advantages over traditional silica-based SAX columns, such as better durability, a wide pH range and the availability of both reversed-phase (styrene-divinyl benzene) and anion exchange retention mechanisms [11], which may be useful for the resolution of non-polar analytes.

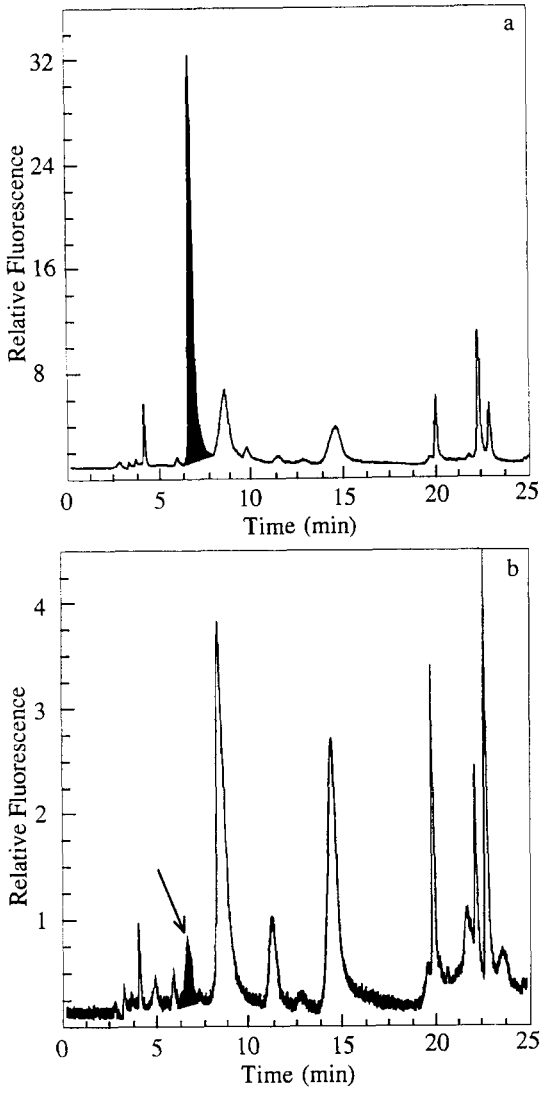


Fig. 3. Typical chromatograms generated from the NDA/CN derivatization of aqueous and cell homogenate samples containing the PNA trimer to form CBI-PNA derivatives. The chromatograms are of an aqueous sample containing the PNA trimer; (a) 816 ng ml$^{-1}$ and (b) 13.87 ng ml$^{-1}$. The chromatographic support consisted of an ODS Hypersil column (5 μm, 250 × 4.6 mm i.d.). A mobile phase composed of acetonitrile-sodium phosphate buffer (pH 2.6, 0.2M) (30:70, v/v) operated at ambient temparature was utilized at a flow rate of 1 ml min$^{-1}$ for these separations. The detailed gradient conditions are described in the Experimental Section.

The mechanism responsible for the retention of the CBI-PNA derivative on the anion-exchange column was determined by delineating the effect of ammonium formate buffer concentration on the retention characteristics of the CBI-PNA derivative. Elution of the derivative was more rapid as the buffer salt concentration was increased, but was unaffected by organic solvent content, suggesting that the PNA-NDA derivative was principally retained by an anion exchange mechanism [11]. Organic solvent content did effect retention of cell matrix interferent peaks and it proved useful to adjust this parameter to achieve separation of these components from the CBI-PNA derivative.

The linearity of the assay and limit of quantitation of the derivatized product in cell homogenate samples were determined using the sample preparation and anion-exchange LC method previously described. Each calibration point was generated by averaging the results of three determinations. From the data generated, a calibration plot could be constructed on the basis of CBI-PNA peak height (PH) with linear regression analysis providing the following equation: CBI-PNA (μM) = 0.076 (μM/cm) [PH (cm)] − 0.060 (μM), $r = 0.999$. The mass limit of detection (MLOD) was determined to be 4.89 ng (prederivatization concentration 48.97 ng ml$^{-1}$). Fig. 4 shows sample chromatograms obtained during the generation of calibration curve and limit of quantitation data.

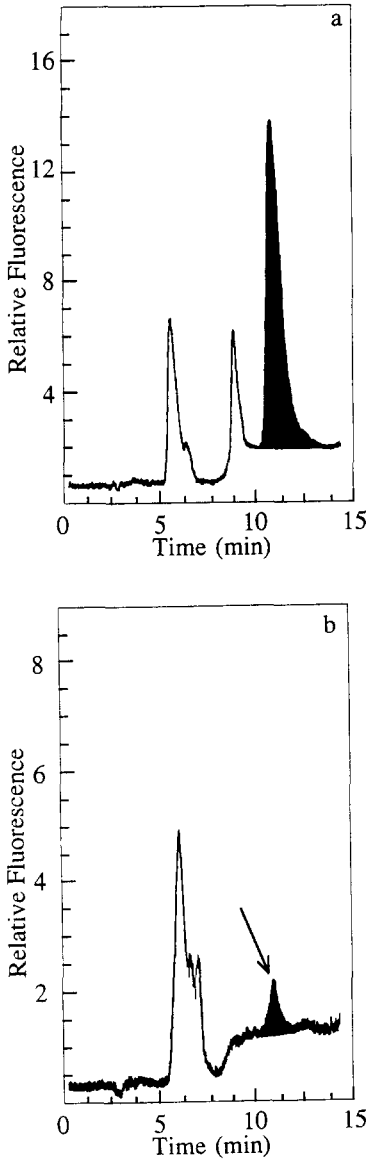


Fig. 4. Cell homogenate sample spiked with (a) 816 ng $ml^{-1}$ and (b) 48.97 ng $ml^{-1}$ PNA trimer. The chromatographic support consisted of Hamilton PRPX 100 strong anion exchange column (250 × 4.6 mm i.d.). A mobile phase composed of acetonitrile-ammonium formate buffer (0.15 N, pH 5.5) (35:65, v/v) operated at ambient temperature at a flow rate of 1 ml $min^{-1}$ for these separations. The gradient conditions are described in the Experimental Section.

## 4. Conclusions

The NDA/CN reagent system forms a highly fluorescent derivative when reacted with PNA trimer and should be generally applicable to larger PNA based structures. The method described for the analysis of PNA in cell homogenates is rapid, reproducible and easily performed and should be adaptable to the analysis of PNA in biological fluids such as plasma and urine.

## Acknowledgements

Authors would like to thank Dr Binghe Wang and Dr Sanjeev Gangwar for their help in the synthesis of PNA trimer. Mark J. Rose acknowledges support from the NCI Training Grant (CA09242-20), Pharmaceutical Research and Manufacturers of America Foundation Inc. and the Procter and Gamble Company.

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