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Characterization of digoxigenin-labeled B-phycoerythrin by capillary electrophoresis with laser-induced fluorescence Application to homogeneous digoxin immunoassay

Fu-Tai A. Chen*, Stephen L. Pentoney, Jr.

Beckman Instruments Inc., 2500 Harbor Boulevard, Fullerton, CA 92634, USA

Abstract

A laser-induced fluorescence (LIF) immunoassay technique based on capillary electrophoretic (CE) separation is demonstrated. The analysis of digoxin in serum at clinically useful concentration levels of 10^{-9} to 10^{-10} *M* is achieved using this technique. The chemistry presented here using digoxigenin-labeled B-phycoerythrin was selected as a convenient model for the exploration of CE–LIF-based immunoassays. The LIF system described here exhibits detection limits in the low 10^{-11} *M* range for several common fluorophores. The data presented in this report are one of the first examples of nanomolar quantitative analysis in a human serum matrix by CE.

1. Introduction

In general, an immunoassay is based on the specific chemical reaction between an antibody and its corresponding antigen. Quantitation involves the separation of antibody-bound antigen from the free antibody or free antigen followed by detection of antibody-bound antigen or free antigen, depending upon the specific analytical scheme employed. In a competitive binding immunoassay, a labeled antigen (the label may be a radioactive isotope, a fluorophore, a chemilumiphore, a simple chromophore or an enzyme) and the antigen of interest compete for an appropriate amount of antibody. Differentiation between bound and free labeled antigen is accomplished by physical, chemical or enzymatic means such as the enzyme multiplied immunoassay technique (EMIT) [1] or fluorescence polarization [2]. Competitive immunoassay is generally suitable for analyte concentrations greater than 10^{-9} *M*. Analytes present at concentration levels below 10^{-9} *M* are generally analyzed by a solid-phase-based sandwich immunoassay [3]. There are many variations of the immunoassay technique, but invariably, the critical step is the separation of bound and free materials.

Capillary electrophoresis (CE) is rapidly becoming one of the most powerful tools for the separation of ionic species such as proteins, peptides, nucleic acids and other water-soluble molecules [4–10]. The utility of CE for routine serum protein analysis [11–13], assay of hemoglobin variants [11], and immunochemical reactions [14] has been demonstrated using photo-

* Corresponding author.

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metric absorbance detection. The primary disadvantage of absorbance detection for CE-based immunoassays is a lack of sensitivity and the many interfering substances present in the serum matrix. In general, absorbance detectors for CE exhibit minimum detectable concentration limits in the mid 10^{-6} *M* range [15]. For many immunoassays this is several orders of magnitude above the required sensitivity levels, thus precluding the use of CE–UV absorbance.

Laser-induced fluorescence (LIF) detection with CE was first introduced by Gassmann *et al.* [16]. Several groups have since demonstrated that LIF detection can extend the sensitivity of CE-based analysis by as much as 6 orders of magnitude, with minimum detectable concentration limits falling in the 10^{-11} to 10^{-12} *M* range [17–19]. In fact CE–LIF has been successfully applied to sensitivity-demanding applications such as DNA sequencing [19–20]. Recently, a competitive immunoassay of insulin by CE–LIF was demonstrated by Schultz and Kennedy [21] with sensitivity of 2 n*M* in a relatively clean system. Shimura and Karger [22] reported a direct immunoassay of human growth hormone by CE–LIF with 0.1 ng/ml sensitivity by isoelectric focusing condition technique. We report here a CE–LIF-based competitive immunoassay for digoxin in human serum.

2. Materials and methods

2.1. Digoxigenin-labeled B-phycoerythrin (D-BP)

In order to isolate B-phycoerthrin in an environment that would support the labeling reaction, a 1.0 mg/ml solution of B-phycocerythrin (Molecular Probes, Eugene, OR, USA) was dialyzed in 20 m*M* phosphate and 75 m*M* sodium chloride at pH 7.0 (PBS). Referring to Fig. 1, the resulting dialyzed B-phycoerythrin was allowed to react with 0.1 mg of digoxigenin-3-O-methylcarbonyl-ε-caproic acid-N-hydroxysuccinimide ester (digoxigenin-NHS-ester, Boeringer Mannheim, Indianapolis, IN, USA) in 100 μl dimethylformamide at 4°C overnight. The reaction mixture was dialyzed in PBS. The concentration of digoxigenin labeled B-phycoerythrin was determined on a Beckman DU-7500 spectrophotometer (λ_{max} = 546 nm, ε: 2 410 000 cm^{-1} M^{-1}). The stock solution of D-BP was diluted in PBS containing 0.05% sodium azide and 0.1% goat serum.

2.2. Immunoassay

An affinity pure Fab fragment isolated from goat antiserum to digoxin was purchased from

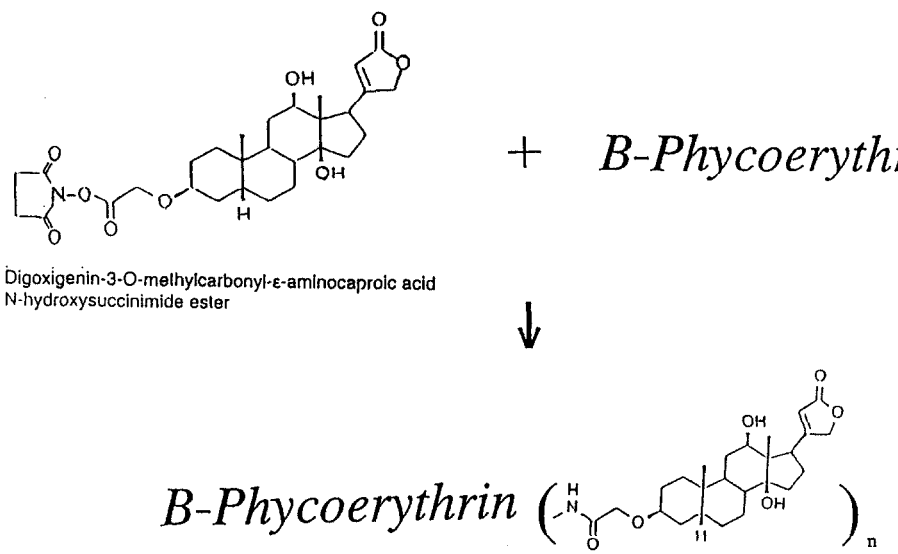

Fig. 1. Synthetic scheme for B-phycoerythrin labeled digoxigenin production.

Boeringer Mannheim. Human serum-based digoxin calibrators at 0.42, 2.72 and 5.21 ng/ml were obtained from Beckman Instruments (Brea, CA, USA). Antibody was diluted in PBS containing 2 mg/ml bovine serum albumin (BSA). Digoxigenin-labeled B-phycoerythrin, 50 μl at 0.5 μg/ml was mixed with 50 μl of serum calibrator and 50 μl of PBS containing 2 mg/ml BSA. Immunoreaction was initiated by the addition of 25 μl Fab antibody (1 μg/ml) and the sample was held at room temperature for 10 min (end-point) [23]. Analysis of immunoreaction products was performed using an automated CE system equipped with LIF detection. Post-run data was analyzed using System Gold software from Beckman Instruments (Fullerton, CA, USA).

2.3. CE–LIF

All CE–LIF work presented here was performed using a modified Beckman P/ACE 2100 CE system. The modification consisted of removing the UV absorbance detector and installing a custom made LIF detector similar to that reported by Pentoney et al. [19]. A 2.0 mW helium neon laser (543.5 nm; Particle Measuring Systems, Boulder, CO, USA) was used to excite B-phycoerythrin and its derivative. The laser output was filtered using a 10 nm laser line filter and was focused into the detection region of the separation capillary using a 5 cm focal length plano convex lens. The fluorescent emission was collected and collimated using a parabolic reflector which held the capillary at its focus. A scatter mask was placed across the front of the parabola in the plane of intense laser scatter. The collimated emission was passed first through a custom made notch filter (543.5 nm blocking), then through a 9 nm band pass filter centered at 580 nm (both filters were purchased from Barr Associates, Westford, MA, USA), and finally onto the photocathode of an end-on type photomultiplier tube (R374; Hamamatsu, Hamamatsu City, Japan).

An uncoated, fused-silica capillary column, typically 37 cm (30 cm to detector window) × 75 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) was used for all CE separations reported here. Approximately 5 nm of the polyamide coating, about 8 cm from the outlet was removed and this region served as the detection window. Electrophoresis was performed at room temperature using 80 m*M*, pH 10.0 borate buffer. The typical voltage gradient used for CE separations was 200 V/cm and the corresponding current was 70 μA. The column was rinsed between runs with 1.0 *M* sodium hydroxide followed by deionized water.

3. Results and discussion

B-Phycoerythrin is one of the fluorescent proteins of the phycobiliprotein family [23] and is both highly water soluble and chemically stable. The absorbance maximum of B-phycoerythrin is at 546 nm with molar absorptivity, ϵ: 2 410 000 cm^{-1} M^{-1} and the quantum yield of B-phycoerythrin is reported to be 0.98 [23]. B-Phycoerythrin is therefore an ideal fluorophore for CE–LIF using the green He–Ne laser. The isoelectric point of B-phycoerythrin is estimated to be 5.5, as a result, it may be readily separated from the antibody by CE using a simple borate based buffer system. Derivatization of B-phycoerythrin with digoxigenin-NHS-ester results in the formation of D-BP (Fig. 2A). Neither the electrophoretic mobility of digoxigenin nor the fluorescence quantum efficiency of B-phycoerythrin appear to be significantly affected by digoxigenin–fluorophore attachment. Analysis of D-BP by CE–LIF at 2.5 ng/ml (equivalent to 10^{-11} *M*, based on the absorption of B-phycoerythrin at 546 nm) indicates that the signal attributable to D-BP is approximately 20 times the baseline noise (data not shown), indicating the excellent lower limits of detection for this LIF system.

Even though B-phycoerythrin possesses multiple amino-tagging sites, D-BP appears to be an electrophoretically homogeneous product by CE–LIF analysis as illustrated in Fig. 2A. This effect is presumably attributable to the large mass difference between the label and the antigen and the large number of charged residues present on the label. As a result, there is little difference in mass to charge ratio for the various multiply labeled products. This observation is

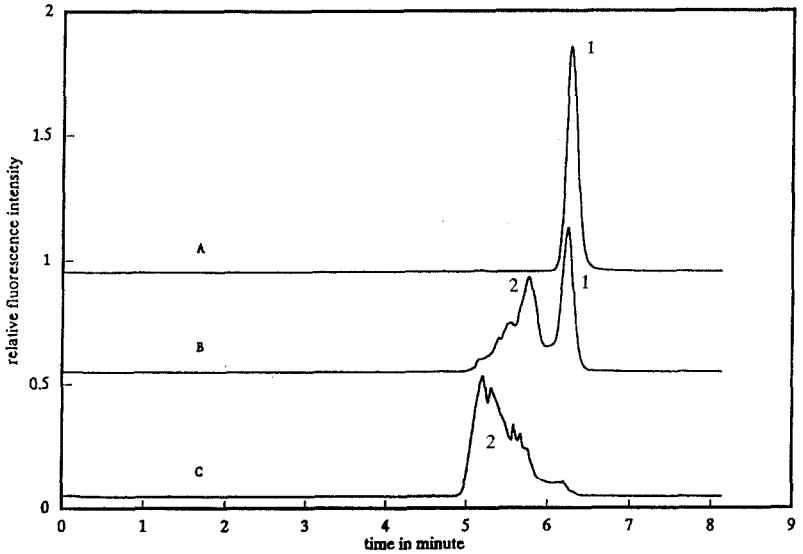

Fig. 2. Electropherogram of CE–LIF of digoxigenin-B-phycoerythrin (D-BP) and its reaction with antibody. Conditions: untreated fused-silica capillary, 35 cm × 75 μm (I.D.); applied potential, 7 kV/70 μA; buffer, 80 m*M* borate at pH 10.0. Peaks: 1 = Antigen–antibody complex of the Fab and D-BP; 2 = D-BP. (A) D-BP: 0.5 μg/ml; (B) reaction mixture between D-BP (0.5 μg/ml/100 μl) and Fab antibody to digoxin (5 μg/ml/10 μl); (C) reaction mixture between D-BP (0.5 μg/ml/100 μl) and Fab antibody to digoxin (10 μg/ml/10 μl).

noteworthy in that the labeling scheme described here utilizes a label which is roughly 600 times greater mass than the analyte. This situation is in direct contrast with most of the reported CE–LIF labeling schemes in which the label and analyte are of comparable size or in which the label is quite small relative to the analyte.

The competitive immunoassay reported here is described by the following equilibrium expression;

$$Ag + Ag^* + Ab \rightleftharpoons Ag\text{–}Ab + Ag^*\text{–}Ab$$

where Ag = antigen, Ag* = labeled antigen and Ab = antibody.

Figs. 2A–D show the separation of reaction mixtures prepared using a fixed concentration of D-BP (Ag*) (0.5 μg/ml) and an increasing amount of Fab antibody (Ab). The existence of D-BP heterogeneity becomes quite apparent when an excess of antibody is added to the reaction mixture. This is illustrated by the multiple peaks shown in Fig. 2C. The heterogeneity becomes apparent in this instance because the number of digoxigenin molecules bound to B-phycoerythrin determines the number of Fab antibody binding sites in the complex. The Fab molecule is roughly 20% of the mass of B-phycoerythrin. As a result, D-BP heterogeneity leads to electrophoretically different mobilities in the bound complex. Note also that there is nearly complete absence of D-BP in the antibody excess–equilibrium mixture, as expected.

Digoxin calibration is demonstrated in Fig. 3A–D with serum-based digoxin calibrators of 0.42, 2.72, 5.21 and 10.42 ng/ml, respectively. As the digoxin concentration is increased in the sample, the amount of free D-BP observed in the electropherogram also increases while the labeled antigen-antibody complex decreases. This trend demonstrates the validity of our method of analysis. Using the ratio of the areas of free D-BP and the total area of Ab ↔ D-BP + D-BP, the digoxin calibration curve presented in Fig. 4 was generated. Both free D-BP and antibody bound D-BP can be measured simultaneously using this assay. Reaction kinetics indicate that the immunoassay reaches an end point in 10 min [24].

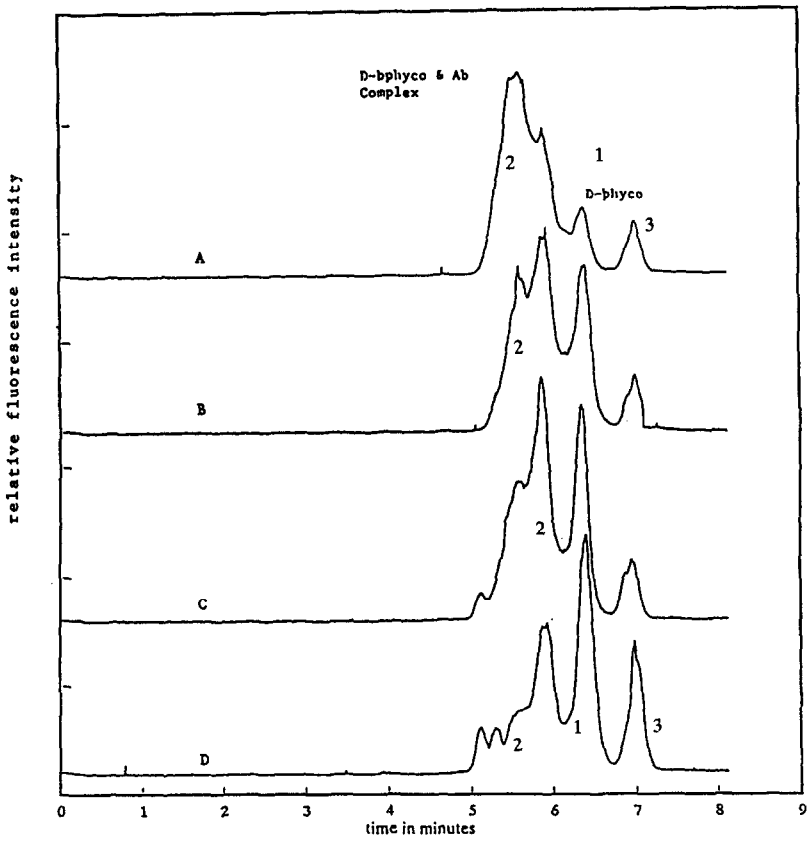

Fig. 3. Electropherogram of CE–LIF of digoxin assay mixture with serum calibrators. Conditions: same as Fig. 2. Peaks: 1 = antigen–antibody complex of the Fab and D-BP; 2 = D-BP; 3 = unknown fluorescence substance derived from serum. (A) Digoxin: 0.42 ng/ml; (B) digoxin: 2.72 ng/ml; (C) digoxin: 5.21 ng/ml; (D) digoxin: 10.42 ng/ml.

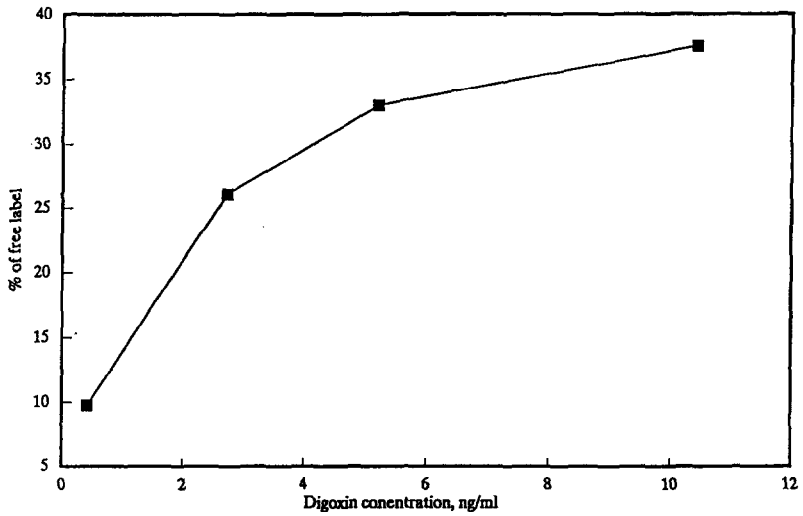

Fig. 4. Calibration curve for digoxin assay by CE–LIF. Percentage of free D-BP vs. digoxin calibrators.

The above procedure is an exploratory attempt to use CE–LIF for competitive immunoassay. The same concept is applicable to a large molecule such as a protein antigen. A monoclonal antibody to an epitope of the protein antigen is required. The epitope, usually a synthetic peptide of 5 to 10 amino acids, may be synthesized and labeled with a fluorophore and used as the competing species for the immunoassay. Direct assay for a protein antigen requires some modification of the antibody. The antibody–antigen complex is expected to have a charge-to-mass ratio near the arithmetic mean of those of the antigen and the antibody. Proper modification of the antibody would provide sufficient electrophoretic resolution between the antibody and the antibody–antigen complex. If the labeled and modified antibody is used in excess with respect to the protein antigen, the amount of complex formation should be directly proportional to the concentration of the protein antigen. Thus, a single antibody species is required to achieve a quantitative immunoassay, instead of the pair of antibody species required for sandwich immunoassays with solid phase separation. The use of excess antibody in solution should provide much more favorable reaction kinetics than found with the traditional solid-phase-based sandwich immunoassay. The combination of chemical modification of the antibody or antigen and separation by CE, coupled with LIF detection may prove to be a useful tool for quantitative immunoassays.

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