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Capillary electrophoretic immunoassays for digoxin and gentamicin with laser-induced fluorescence polarization detection

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Abstract

New immunoassays for therapeutic drugs digoxin and gentamicin have been described, which involved the separation of free and antibody-bound drug by capillary electrophoresis (CE) and the detection by laser-induced fluorescence polarization (LIFP). While the fluorescein-labeled digoxin and gentamicin (tracers) displayed negligible fluorescence polarization in solution, the complex formation between these small molecules and their antibodies resulted in substantial increases in fluorescence polarization due to the increase in molecular size. The LIFP detection, capable of measuring vertically and horizontally polarized fluorescence components simultaneously, provides enhanced capability for the identification of complex in capillary electrophoretic immunoassays. Proper adjustments of the running buffer pH and the ratio of antibody to tracer are essential for optimization of the performance of these assays. The digoxin–antibody complex remained stable during CE separation with running buffer pH ranging from 9.3 to 12. Calibration curves covering a concentration range of 0.05 to 0.5 ng/ml were obtained with a running buffer of pH 12. The concentration and mass detection limits were 0.02 ng/ml and 26 zmol, respectively. For gentamicin assay, the running buffer pH 10 was used to reduce the adsorption of the tracer while minimizing the dissociation of the antibody–tracer complex during the separation. The calibration curves covered a concentration range 0.05–1.0 μg/ml, with a concentration detection limit of 25 ng/ml and a mass detection limit of 52 amol of gentamicin.

Keywords: Immunoassays; Digoxin; Gentamicin

1. Introduction

Capillary electrophoretic immunoassay (CEIA) has emerged as an attractive alternative to many conventional assay techniques [1–5]. When combined with sensitive detection schemes such as laser-induced fluorescence (LIF), CEIA offers several advantages including high mass detection sensitivity, low sample and reagent consumption, and being amenable to miniaturization and automation. It has been successfully applied to the determination of a variety of pharmaceutical compounds including insulin [6], human growth hormone [7], chloramphenicol [8], morphine [9], cortisol [10], theophylline [11,12], digoxin [13], and cyclosporine [14]. All these applications rely on separations of the free and

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bound tracer for the identification and quantitation. When the two species are not resolvable by CE, however, the technique is not applicable and is unable to reveal the formation of the antibody–tracer complex. Recently, we have shown that substituting LIF with laser-induced fluorescence polarization (LIFP) provides additional means to confirming immunocomplex formation and can expand the applicability of CEIA to include both heterogeneous and homogeneous assays [15].

Fluorescence polarization is associated with rotational motion of a fluorescent molecule [16–18]. When fluorescent molecules in solution are excited with a polarized light, they will emit partially polarized fluorescence. The degree of such polarization is proportional to the molecule's rotational correlation time. Upon excitation, small molecules rotate fast during the excited state and thus have low fluorescence polarization whereas large molecules rotate slowly and exhibit larger fluorescence polarization. This is the basis of homogeneous fluorescence polarization immunoassays involving competition of analyte with fluorescently labeled tracer for binding to the limiting amount of a specific antibody [19–21]. We reported the use of LIFP detection for CEIA of cyclosporine [14]. We now demonstrate the application of the CE–LIFP approach to the determination of digoxin and gentamicin.

Development of sensitive and selective assays for therapeutic drugs digoxin and gentamicin is of considerable importance in clinical practice. Digoxin is a cardiac glycoside prescribed for treatment of congestive heart failure and supraventicular arrhythmias, and gentamicin is an aminoglycoside antibiotic used to treat infections caused by Gram-negative bacteria [22,23]. Because of their narrow therapeutic ranges (0.8–2.0 ng/ml for digoxin and 5–10 μg/ml for gentamicin), frequent monitoring of the serum drug levels of individual patients is required to avoid toxic side effects and to achieve optimum therapeutic response. Preliminary results showed that the CE–LIFP approach is ideally suited for digoxin assay but needs further optimization for gentamicin assay. The attractive features of this approach include high mass detection sensitivity, low sample consumption, enhanced capacity for sample identification and the potential for further miniaturization.

2. Experimental

2.1. Instrumentation

The CEIA–LIFP studies were performed using an experimental set-up similar to that described previously [14]. A high-voltage power supply (Model CZE 100R, Spellman, Plainview, NY, USA) was used to drive sample through a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The detection end of the capillary was inserted in a sheath flow cuvette (NSG Precision Cells, Farmingdale, NY, USA), which was grounded. A polarized laser beam from a 65-mW argon ion laser (Model 2014-65ML, Uniphase, San Jose, CA, USA), filtered through a laser line filter (488 nm, 10-nm band width, Newport, Fountain Valley, CA, USA), was used as the excitation source. Fluorescence was collected at 90° with respect to the incident beam by using a 60× microscope objective lens (0.7 NA, Universe Kogaku, Oyster Bay, NY, USA). After passing through a narrow bandpass filter (515 nm, 10-nm band width, Newport) and a pinhole, the fluorescence was separated into vertically (I_v) and horizontally (I_h) polarized components by a polarizing cube beamsplitter (Melles Griot, Irvine, CA, USA). The fluorescence intensities corresponding to these two polarized components were measured simultaneously by two photomultiplier tubes (PMTs, R1477, Hamamatsu, Japan). Instrument operation and data acquisition were controlled through a Power Macintosh computer with application software written in LabView (National Instruments, Austin, TX, USA).

2.2. Materials and reagents

Disodium fluorescein of purified grade was obtained from Fisher Scientific (Fair Lawn, NJ, USA). FPIA reagent set for digoxin, which contains a fluorescein labeled digoxin as the tracer and polyclonal antibody (rabbit) against digoxin, was obtained from Abbott Labs. (Chicago, IL, USA). FPIA reagent set for gentamicin, which contains a fluorescein labeled gentamicin as the tracer and polyclonal antibody (rabbit) against gentamicin, was obtained from Sigma (St. Louis, MO, USA). Exact com-

positions and concentrations of the tracer and antibody solutions from these commercial sources were not disclosed. Serum-based digoxin and gentamicin calibrator sets and FPIA dilution buffer were obtained from Sigma. Serum specimens from patients under digoxin therapy were provided by the Department of Laboratory Medicine, University of Alberta.

2.3. Antibody–tracer complex

Various volumes (0, 4, 6 and 10 μl) of antibody solution and 10-μl aliquots of tracer solution from the FPIA reagent set for digoxin were mixed in 0.5-ml microcentrifuge tubes. FPIA dilution buffer was added to each tube to bring a total volume to 100 μl. The tubes were vortexed for 30 s. The mixtures were allowed to incubate at room temperature for 15 min prior to CE–LIFP analysis. The binding experiments for gentamicin were carried out similarly, with the use of corresponding gentamicin tracer and antibody.

2.4. Competitive immunoassay

Into microcentrifuge tubes were pipetted 6-μl aliquots of digoxin standard solutions (0, 0.5, 1.0, 2.0, 3.0 and 5.0 ng/ml) from its FPIA calibrator set or serum specimens from patients, 6 μl of antibody solution and 8 μl of tracer solution from the reagent set, and 60 μl of FPIA dilution buffer. After 15 min of incubation at room temperature, each sample was analyzed by CE–LIFP. Assays for gentamicin were carried out in the same way as for digoxin, except with a series of gentamicin standard solutions (0, 0.5, 1.5, 3.0, 6.0 and 10.0 μg/ml), gentamicin tracer, and antibody to gentamicin.

2.5. CE–LIFP

A capillary of 35 cm×20 μm I.D.×148 μm O.D. was used for separation. The capillary was conditioned periodically by successive rinsing with 0.1 *M* NaOH, ionized water and the running buffer to ensure the reproducibility of the separation. Samples were electrokinetically injected into the capillary by applying 5 kV for 5 s. Separation was carried out at an applied field strength of 714 V/cm.

The performance of the LIFP detector was optimized by carefully aligning the laser beam with the sample stream and the collection optics and by balancing signals from the two PMTs. During the aligning process, an aqueous solution of disodium fluorescein was constantly introduced into the separation capillary. The laser beam was focused onto a spot about 20 μm below the tip of the capillary that was inserted in the sheath flow cuvette [24–26]. The sheath fluid, identical to the run buffer, was introduced into the cuvette hydrodynamically by keeping the inlet reservoir of the sheath buffer 1 cm higher than the outlet reservoir. The angle and position of the cuvette relative to the detection optical path were adjusted to obtain nearly equal signals with optimum output from both PMTs. The values for fluorescence polarization, *P*, were calculated according to

$$P = (I_v - I_h)/(I_v + I_h)$$

where I_v and I_h are the intensities of the vertically and horizontally polarized components of the fluorescence.

3. Results and discussion

3.1. Assay for digoxin

A common problem in CE-based immunoassay is the possible adsorption of antibody to capillary walls, which can cause peak broadening and poor separation. The use of the running buffers of high pH or ionic strength [6,27–29] has been recommended to alleviate this problem. However, extreme pH conditions may result in the dissociation of the immunocomplex. Thus, the effect of the buffer pH on the stability of the complex was studied. Fig. 1 shows electropherograms obtained with the running buffer pH ranging from 9.3 to 12.0 for mixtures containing fluorescent dye, fluorescent digoxin tracer and antibody against digoxin. Over the pH range investigated, three species corresponding to fluorescent dye, free and antibody bound tracer were well resolved with decreasing migration times. The digoxin tracer–antibody complex appeared to be stable as nearly baseline separation of the free and bound

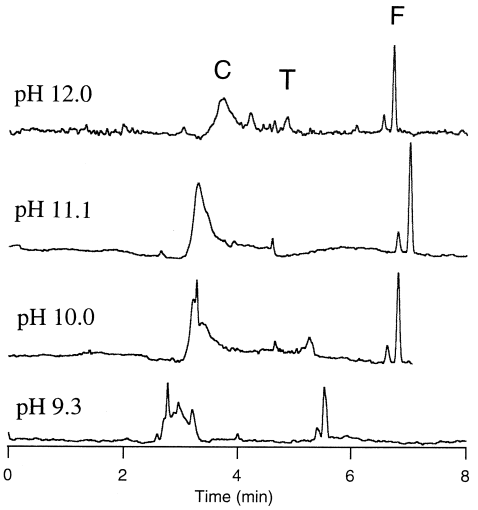

Fig. 1. Effect of the running buffer pH on the separation of a mixture containing digoxin tracer and anti-digoxin antibody. Conditions: separation capillary, 35 cm×20 μm I.D.; running buffer, 10 m*M* disodium tetraborate buffer, pH adjusted with 0.1 *M* NaOH; applied voltage, 20 kV; excitation wavelength, 488 nm; emission wavelength, 515 nm. Only vertically polarized fluorescence component is shown although horizontally polarized fluorescence was simultaneously measured. T, C and F denote digoxin tracer, antibody–tracer complex and fluorescein, respectively.

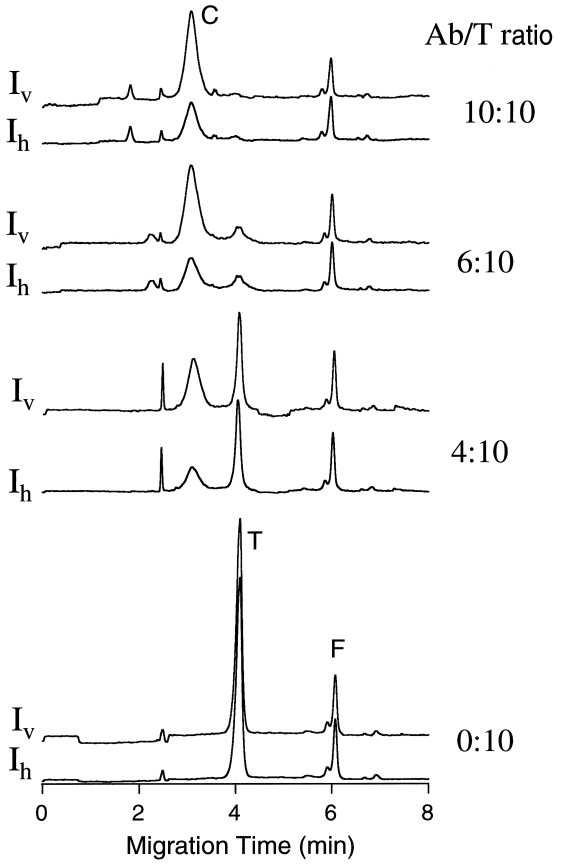

Fig. 2. Electropherograms showing separations of mixtures containing digoxin tracer (T) and its antibody (Ab) at varying volume ratios. Conditions as in Fig. 1 except that the running buffer, pH 12 was used. The traces I_v and I_h correspond to vertically and horizontally polarized fluorescence components, respectively. Peak identities as in Fig. 1.

tracer was obtained in all cases. It is noticeable that the peakshape for the complex was improved considerably upon increasing the pH of the running buffer. A broad and split peak at pH 9.3 was turned into a broad but symmetric peak at pH 12. There is a general increase in migration time with the running buffer pH, which was attributable to a decrease in electroosmotic flow because of the use of higher salt concentrations. These results demonstrate that a proper adjustment of the running buffer pH is a simple yet effective way to reduce the adsorption of the antibody, consequently leading to an improvement in separation efficiency.

The identities of the electrophoretic peaks shown above were established using titration experiments and fluorescence polarization. Fig. 2 shows the variations of peak intensity and fluorescence polarization with increasing antibody/tracer (Ab/T) ratios. First, the addition of the antibody to the digoxin tracer solution causes a decrease in the tracer signal and the appearance of a new peak, which was assigned to the complex. As the Ab/T ratio increases, the intensity of the new peak increases at the expense of the tracer peak, indicating the formation of the complex. A confirmation of the complex formation is provided by the fluorescence polarization information that is also shown in Fig. 2. According to fluorescence polarization theory, an increase in polarization is expected when the digoxin (M_r 781) tracer is bound to its antibody (M_r ~ 150 000) as a result of a substantial increase in the hydrodynamic volume of the fluorescent species and the corresponding decrease in the molecular rotation. Based on the peak heights measured for the fluores-

cent species and normalized against that of fluorescein internal standard, the P values of 0.06 ± 0.02 ($n=6$) and 0.28 ± 0.02 ($n=6$) were found for the tracer and complex, respectively. As an intrinsic molecular property, the P value is characteristic for a given compound or complex. Since electrophoretic migration time and fluorescence polarization are unrelated, the ability to measure the fluorescence polarization of an eluting compound ''on the fly'' during capillary electrophoresis permits complementary information about the identity of the analyte to be obtained instantly.

Quantitative aspects of the competitive assay for digoxin were studied. To this end, a mixture of digoxin tracer and antibody at a fixed ratio was titrated with a series of serum based digoxin standard solutions. CE separation for this series of mixtures was carried out with a running buffer of pH 12. The peak height from LIFP detection of the free and bound tracer normalized against that of fluorescein was used to establish the calibration curves for serum digoxin. Fig. 3 shows four calibration curves obtained corresponding to, respectively, vertically and horizontally polarized fluorescence from the free and bound tracer. As expected from the principle of competitive immunoassays, an increase of digoxin in sample solution leads to an increase in the signal for the free tracer and a corresponding decrease in the signal for the antibody-bound tracer. Although each of the four calibrations can be used for quantitative analysis, those based on free tracer measurements are more sensitive because of their steeper changes with respect to sample concentration. A detection limit for this assay, defined as the sample concentration equivalent to the blank signal plus three-times the standard deviation of replicate blank measurements, was found to be 0.02 ng/ml or 26 pM of digoxin. This concentration detection limit corresponds to a mass detection limit of 26 zmol for a typical injection volume of 1 nl.

The CE–LIFP approach described above was used to assay seven serum samples from patients receiving digoxin therapy. The concentrations of digoxin were calculated from the calibration curve based on the vertical emission of the free tracer. Fig. 4 compares the results with those obtained by an alternate immunoassay method known as DGNA (Dade Behring, Newark, DE, USA). The methodology for DGNA involves mixing antibody enzyme conjugate reagent with patient's serum, magnetic separation of unbound antibody conjugate and colorimetric determination of substrate [30]. The computed line of

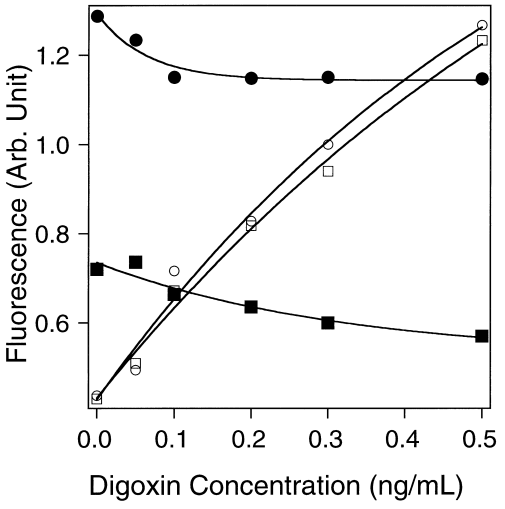

Fig. 3. Calibration curves for digoxin. Conditions as in Fig. 2. (□) Free digoxin tracer, horizontally polarized fluorescence; (○) free digoxin tracer, vertically polarized fluorescence; (■) complex, horizontally polarized fluorescence; and (●) complex, vertically polarized fluorescence.

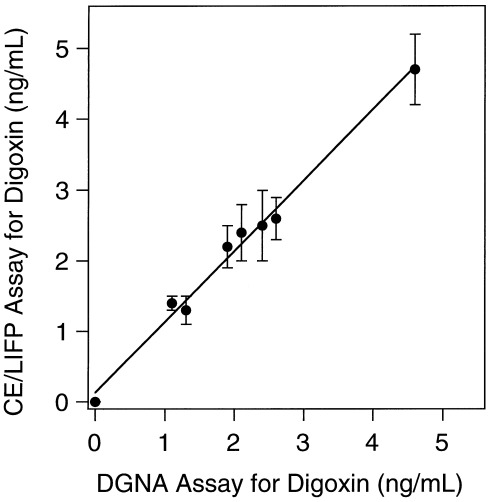

Fig. 4. Correlation between digoxin levels in patients' serum samples as measured by CE–LIFP and DGNA.

best fit ($y=0.24+0.96x$, $R=0.99$, $n=28$) points to a good correlation between digoxin levels in patients' specimens as determined by CE–LIFP and DGNA.

3.2. Assay for gentamicin

The effect of running buffer pH on the migration behavior of fluorescein labeled gentamicin and its complex with antibody was examined and the results are shown in Fig. 5. Over a range of pH 9.3 to 12, the gentamicin tracer displays either a broad peak or multiple peaks. This behavior is probably attributed to heterogeneous, multiple labeled gentamicin. Gentamicin contains three amino groups that can react with fluorescein dye in the labeling reactions. The gentamicin tracer is therefore composed of fluorescein conjugates with the ratio of gentamicin to dye varying from 1 to 3. The conjugates of low gentamicin/dye ratios may be responsible for a broad peak at the lower pH (9.3), due to strong interactions of unreacted amines with capillary walls. At the higher pH (11–12), the ionization of unreacted amino groups in the tracer is suppressed, leading to a reduction in the adsorption of the tracer to the capillary and in turn a better resolution of the multiple labeled conjugates.

The electropherograms shown in Fig. 5 suggest that the stability of the gentamicin tracer–antibody complex is strongly dependent on the pH of the running buffer. Increases in the buffer pH result in decreases in the complex peak and increases in the tracer peak. At pH 12, the complex peak disappears from the electropherogram, indicating a complete dissociation of the antibody–tracer complex. The running buffer pH 10 was found to be a compromise, at which the adsorption of the tracer and the dissociation of the complex were minimal.

Fig. 6 shows separations of mixtures containing

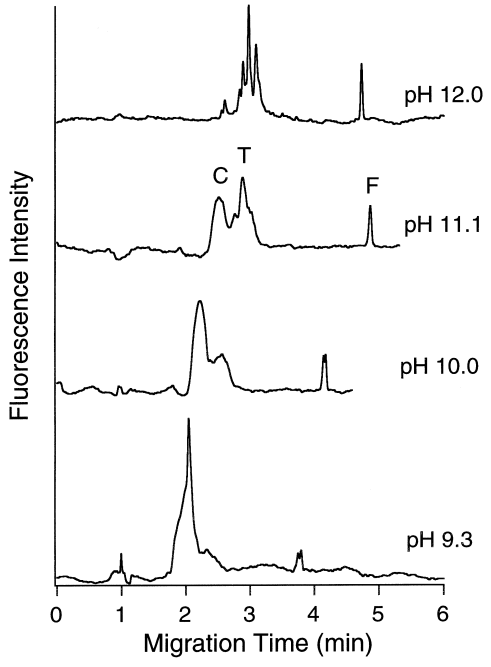

Fig. 5. Effect of the running buffer pH on the separation of a mixture containing the gentamicin tracer and anti-gentamicin antibody. Conditions as in Fig. 1. T, C and F denote gentamicin tracer, antibody–tracer complex and fluorescein, respectively.

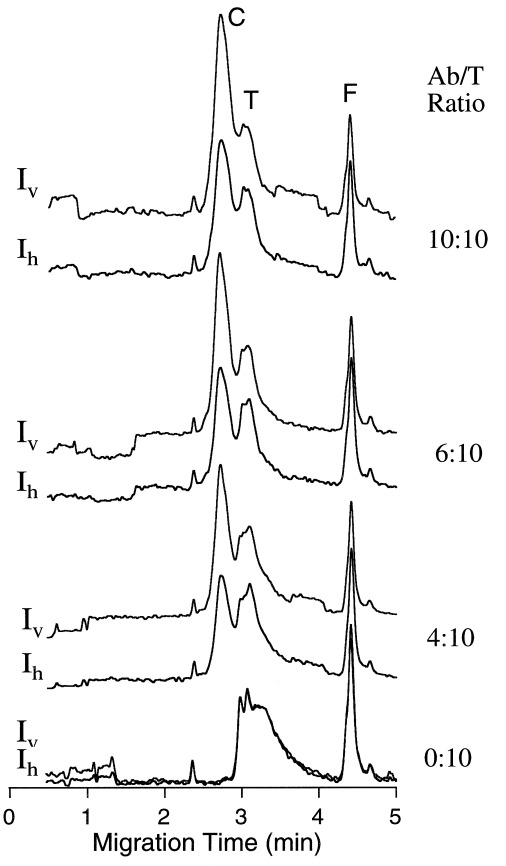

Fig. 6. Electropherograms showing separations of mixtures containing gentamicin tracer (T) and its antibody (Ab) at varying volume ratios. Conditions as in Fig. 1 except that the running buffer, pH 10.0 was used. Peak identities as in Fig. 5.

gentamicin tracer its antibody at varying antibody–tracer ratios. As expected for immunocomplexation, the addition of the antibody leads to increases in the complex peak and the corresponding decreases in the tracer peak. The identities of the peaks were further confirmed by the *P* values which were determined to be 0.07 ± 0.02 ($n=6$) for the tracer and 0.25 ± 0.02 ($n=6$) for the complex. The *P* value for the complex reported here is reasonably close to a literature value of 0.21 [31].

Fig. 7 shows four calibration curves obtained using serum-based gentamicin calibrator set containing 10 to 1000 ng/ml gentamicin. Changes of fluorescence intensity with the gentamicin concentration in sample are steeper with the complex peak than with the tracer peak. A concentration detection limit for gentamicin assay was 25 ng/ml or 52 n*M*. The corresponding mass detection limit was 52 amol of gentamicin for an injection volume of 1 nl.

The utility of the CE–LIFP approach as described here is rather limited because of the poor resolution between free and antibody bound tracer and tendency for the complex to dissociate at high pH. Further optimization of the separation is required before the method can be used for quantitative measurements of gentamicin in patients' samples.

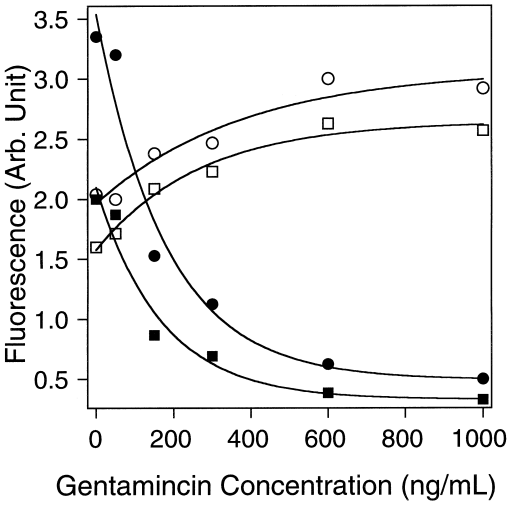

Fig. 7. Calibration curves for gentamicin. Conditions as in Fig. 5. (□) Free gentamicin tracer, horizontally polarized fluorescence; (○) free gentamicin tracer, vertically polarized fluorescence; (■) complex, horizontally polarized fluorescence; and (●) complex, vertically polarized fluorescence.

4. Conclusions

A CEIA–LIFP method for determination of therapeutic drugs has been described and optimized using digoxin and gentamicin as the model systems. The complex of a fluorescently labeled drug (tracer) with an antibody is identified by its characteristic fluorescence polarization. The assay is based on the competition between drug analyte and the tracer for binding to the limiting amount of antibody. This method allows rapid determination of the drug in the nanomolar concentration range with a typical injection volume of 1 nl. The adsorption of antibody or tracer to the capillary walls can be a major factor limiting the performance of this approach because it causes peak broadening and poor reproducibility of the separation. The use of running buffers of high pH combined with periodic rinsing of the capillary can reduce adsorption effectively, leading to an improvement in the separation. However, the usefulness of high pH buffers may be limited by the dissociation of the immunocomplex. For this reason, other approaches such as the use of internally coated capillaries [6,10] and antibody fragments [6,7,10] may be explored to provide viable alternatives.

Acknowledgements

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