Enzyme-Linked Competitive Binding Assays for Digoxin

Moon Hee Choi, Moon Hwan Kim, Ho Choll Cho, Min Sun Kim, Eun Ah Lee,[†] Insook Rhee Paeng,[†] and Geun Sig Cha[*]

Department of Chemistry, Kwangwoon University, Seoul 139-701, Korea
[†]Department of Chemistry, Seoul Women's University, Seoul 139-774, Korea
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Enzymes have emerged as the most powerful alternatives to radioisotopes in the development of binding assay methods for the selective detection of various physiological, biological and environmental substances at trace levels.[1-6] Such methods may be classified as either heterogeneous (solid-phase) or homogeneous (separation-free). The heterogeneous arrangements such as the enzyme-linked immunosorbent assay (ELISA) are much slower, but prevalent. The homogeneous types such as the enzyme-multiplied immunoassay technique (EMIT) are much faster because there is no need for separation of free and bound enzyme labels.[5,6] In this method, analytical signals result typically from inhibition of enzyme-conjugate catalytic activity in solution by antibody (or binding protein) interactions with the conjugate.

We have previously devised a generic type of the homogeneous method based on the strong and specific biotin/avidin interaction for the detection of biomolecules other than biotin.[7-10] In this method, the binding reaction between the enzyme-biotin and avidin-analyte conjugates inactivates the enzyme. In the presence of analyte-specific binder, the enzymatic activity of the conjugate is regained (*i.e.*, less inhibited) since the binding of the binder to the avidin-analyte conjugate prevents the enzyme inactivation by sterically hindering the binding between the avidin-analyte and enzyme-biotin conjugates. The biotin/avidin interaction may also be utilized in devising a heterogeneous assay protocol. For instance, the enzyme-biotin conjugate can serve as a signal generator by binding to the avidin-analyte conjugate that has already been bound to the analyte-specific binder immobilized on a solid surface.

The aim of this work is to compare the analytical performance of several different enzyme-linked competitive binding assay methods using a monoclonal anti-digoxin antibody as a model binder: *i.e.*, homogeneous versus heterogeneous assays, and conventional EMIT or ELISA versus biotin/avidin-mediated techniques. In this work, we further investigated the feasibility of developing an assay method useful for the determination of serum digoxin. Digoxin is the most widely used cardiac glycoside for treating congestive heart failure.[11,12] Because of a narrow therapeutic range (*i.e.*, 0.8-2.0 ng/mL), serum digoxin levels are frequently determined throughout theraphy.[13] In this work, three different enzymes are examined as labels: *i.e.*, glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase (MDH), and alkaline phosphatase (ALP). Enzyme conjugates are evaluated in both heterogeneous and homogeneous assay protocols by comparing the detection capabilities of the resulting assay systems. The relative advantages and disadvantages of each assay protocol are discussed based upon the findings of this work.

Experimental Section

Reagents. Glucose-6-phosphate dehydrogenase (G6PDH) from Leuconostoc mesenteroides, malate dehydrogenase (MDH) from porcine heart mitochondrial, alkaline phosphatase (ALP) from bovine intestinal mucosa, glucose-6-phosphate (G6P), β-nicotinamide adenin dinucleotide (NAD), β-dihydrodiphosphopyridin nucleotide (NADH), oxaloacetate, p-nitrophenyl phosphate, bovine serum albumin (BSA), N-hydroxysuccinimidobiotin (NHS-biotin), biotinamidocaproyl-labeled ALP (ALP-biotin), avidin from egg white, and digoxin were obtained from Sigma (St. Louis, MO, USA). Digoxigenin-3-0-methylcarbonyl-μ-aminocaproic acid-N-hydroxysuccinimide ester (NHS-digoxin) was purchased from Boehringer Mannheim (Mannheim, Germany). Monoclonal anti-digoxin antibody (with an association constant of about 4×10^{11} M^{-1}) and an avidin-digoxin conjugate (with an average number of 30 digoxin molecules per avidin molecule) were graciously provided by Dade Behring Inc. (Newark, DE, USA).

The assay buffers used for the homogeneous assays with G6PDH, MDH, and ALP conjugates were as follows: 0.05 M Tris-HCl (pH 7.8), 0.1 M sodium phosphate (pH 7.5), and 0.05 M sodium carbonate (pH 9.5), respectively, containing 0.1 M NaCl and 0.01% (w/v) NaN_3, 0.01% gelatin (w/v) and 0.2% or 2% (for MDH) BSA (w/v). Dilutions of conjugates, binders, standards, and sample solutions were made using this assay buffer. The buffer solutions used for the heterogeneous assays with ALP conjugates were as follows: 0.05 M Tris-HCl (pH 7.4) containing 0.02% NaN_3, 0.5% BSA for dilutions and binding reactions, 0.05 M Tris-HCl (pH 7.4) containing 0.05% NaN_3 for washing, and 0.05 M sodium carbonate (pH 9.5) for activity measurements, respectively.

Preparation of Conjugates. Enzyme conjugates were prepared by reacting each enzyme with NHS-biotin or NHS-

[*]Corresponding Author. e-mail: gscha@daisy.gwu.ac.kr

digoxin according to the N-hydroxysuccinimide ester method, as described previously.[7-10,14,15] For the preparation of G6PDH-digoxin conjugates, the required amount of NHS-digoxin dissolved in DMF was added to 500 μL of coupling buffer (0.1 M bis-tris propane, pH 6.5) containing a given amount of G6PDH (200 units). The active site of G6PDH was protected during the conjugation reaction by adding an excess of G6P and NADH. In the case of MDH- and ALP-digoxin conjugates, 500 units of MDH or 200 units of ALP was dissolved in 500 μL of 0.1 M sodium phosphate, pH 7.5 or 0.05 M sodium carbonate, pH 9.5, respectively. The reaction was run for 24 hr at 4 °C under stirring. The reaction mixture was then dialyzed against 0.05 M Tris-HCl, pH 7.4 for G6PDH- and ALP-digoxin or 0.1 M sodium phosphate, pH 7.5 for MDH-digoxin, and was diluted to a final volume of 2.0 mL with the dialysis buffer.

The resulting enzyme conjugates were characterized by their residual activities and percent inhibitions induced by an excess amount of the corresponding binder (*i.e.*, avidin or anti-digoxin antibody);[7-10,14,15] see Table 1. All conjugates were kept at 4 °C until the additions of reagents for activity measurements.

Homogeneous Assays. The rate of increase of NADH measured by the change in absorbance at 340 nm per unit time, decrease of NADH at 340 nm or increase of *p*-nitrophenol at 405 nm was used to determine the activity of G6PDH,[7] MDH[15] or ALP[7,8,10] conjugates, as described previously. In order to obtain data for the maximum percent inhibition, each enzyme conjugate solution was added to an assay tube containing an excess amount of the corresponding binder (*i.e.*, anti-digoxin antibody or avidin), and the resulting activity was measured.[7,8,10,15] For EMIT-type homogeneous assays employing G6PDH and MDH conjugates, digoxin standards were incubated first with anti-digoxin antibody, and then with enzyme conjugates before the activity measurements as described in earlier works.[15] In the biotin/avidin-mediated assay protocol for digoxin, digoxin standards were incubated with anti-digoxin antibody, avidin-digoxin conjugate, and with ALP-biotin conjugate, subsequently, before the activity measurements.[10]

Heterogeneous Assays. Heterogeneous digoxin assays were performed by first immobilizing anti-digoxin antibody on the inner surface of microwells by physical adsorption.[10]

Table 1. Characteristics of Enzyme Conjugates

Conjugate	Initial ratio (NHS-analyte/enzyme)[a]	% Residual activity	% Inhibition[b]
G6PDH-digoxin	50	48	54
MDH-digoxin	50	36	54
ALP-digoxin 1	50	83	7
ALP-digoxin 2	2500	43	20
ALP-biotin 1	2250	44	90
ALP-biotin 2[c]	–	–	20

[a]Refers to molar ratios of NHS-digoxin or NHS-biotin to enzyme during the conjugation reaction. [b]For all conjugates, a fixed amount of enzyme-analyte conjugate was incubated with a fixed amount of the binder. [c]Purchased from a commercial source.

For the conventional ELISA-type protocol, anti-digoxin antibody-coated well were incubated with digoxin standards, washed, and incubated with ALP-digoxin before activity measurements, as described previously.[10] In the biotin/avidin-mediated heterogeneous assay protocol, 100 μL of digoxin standards were added to each anti-digoxin antibody-coated well, and incubated for 2 hr. After washing, 100 μl of an avidin-digoxin conjugate solution was added and incubated for 2 hr. After washing, 100 μL of an ALP-biotin conjugate solution was added and incubated for 2 hr. The wells were then washed and filled with 100 μL of 10 mM *p*-nitrophenyl phosphate dissolved in 1.0 M diethanolamine (pH 9.8) containing 0.5 mM magnesium chloride. After incubation for 30 min at room temperature, the absorbance of the wells was determined with an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

Results and Discussion

For the sensitive enzyme-linked assay development, the choice of an appropriate enzyme-analyte conjugate is very essential. An ideal enzyme-analyte conjugate for homogeneous assays should be inhibited to a large degree by an excess of binder, and yet should retain a useful residual activity. On the other hand, a less inhibited enzyme conjugate is desired for use in heterogeneous type assays. Therefore, various enzyme conjugates were prepared by reacting each enzyme with different amounts of NHS-digoxin or NHS-biotin. In general, higher initial NHS-analyte/enzyme molar ratios used during the conjugation reaction yielded greater conjugate inhibition by a given excess of the binder. In this work, a greater inhibition was achieved at the expense of a decrease in the residual activity of the conjugate as is typical with most enzyme conjugate systems.

Table 1 summarizes the parameters characterizing the conjugates examined in this study, particularly those of the most highly inhibited conjugate for each enzyme system and of the ALP conjugates employed for heterogeneous assays. For the G6PDH- and MDH-digoxin conjugate systems, we were able to make a highly inhibited conjugate in both cases, *i.e.*, up to 54% by an excess of anti-digoxin antibody, as can be seen in Table 1. In the case of ALP-digoxin, however, only 20% of the maximum percentage inhibition was possible even with the use of a high initial NHS-digoxin/ALP ratio (*i.e.*, 2500) during the conjugation reaction. These observations demonstrate that conjugates based on two-substrate enzymes (*i.e.*, G6PDH and MDH) are more easily inhibited by the binding reaction than those based on single substrate enzymes (*i.e.*, ALP). It is believed that the activity of the enzyme conjugate having only one substrate site is less susceptible to steric hindrance or conformational change induced by the binding of the binder to the conjugate, and thus, is less inhibited upon the binding reaction than that of the enzyme conjugate with two substrate sites. Weakly inhibited ALP-digoxin conjugates may be useful for the heterogeneous arrangements, but are not desired for the develop-

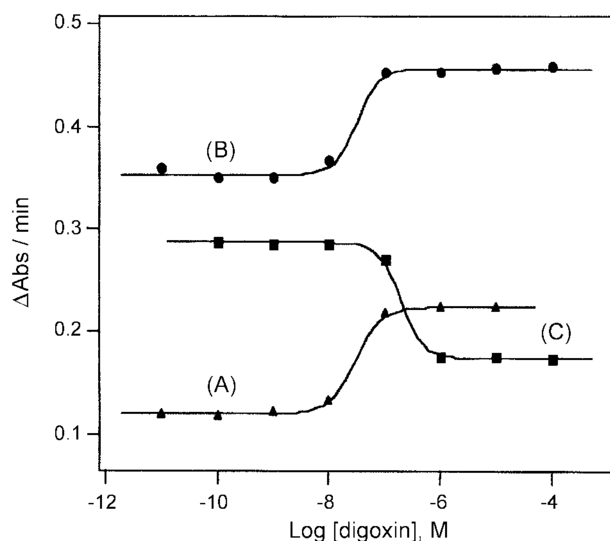

Figure 1. Dose-response curves obtained with homogeneous assays for digoxin: A) 0.17 units of G6PDH-digoxin, and 1 μg of anti-digoxin antibody; B) 0.25 units of MDH-digoxin and 1 μg of antibody; and C) 0.36 units of ALP-biotin, 6.25 μg of antibody and 1.25 μg of avidin-digoxin.

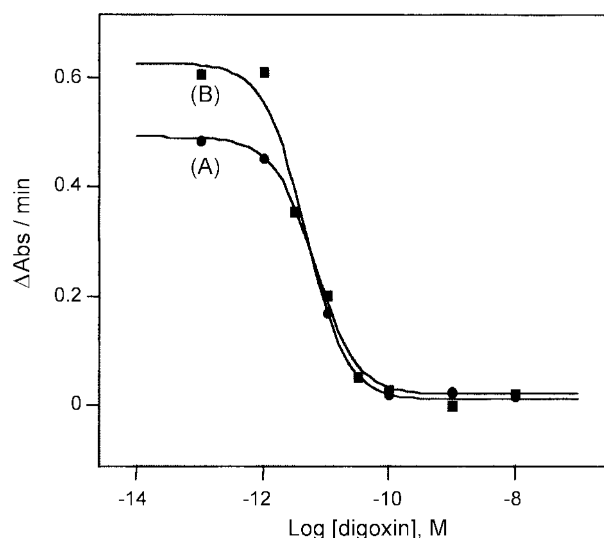

Figure 2. Dose-response curves obtained with heterogeneous assays for digoxin: A) 0.02 units of ALP-digoxin 1 and 0.05 μg of antibody; B) 0.0752 units of ALP-biotin, 0.05 μg of antibody and 0.4 μg of avidin-digoxin.

ment of a sensitive homogeneous assay. This is because the analytical signal of the homogeneous assay comes from inhibition of enzyme activity by binder interaction with the conjugate. For this reason, the ALP-digoxin conjugates were not further examined for homogeneous assays in the subsequent experiments. On the other hand, the ALP-biotin conjugates were inhibited to a very high degree, up to 90%, by an excess of the binder protein, avidin (see Table 1). This may be explained by an extremely high affinity ($K_a = 10^{-15}$ M) of the biotin/avidin reaction and the quadravalency of avidin.[16]

Figure 1 compares dose-response curves for digoxin obtained with different homogeneous assay protocols: A) EMIT-type with G6PDH-digoxin, B) EMIT-type with MDH-digoxin, and C) biotin/avidin-mediated assay with ALP-biotin and avidin-digoxin. The three different assays were optimized and their reagent concentrations were adjusted to produce similar analytical signals (*i.e.*, absorbance change per minute). As can be seen, the G6PDH- and MDH- based EMIT-type assays exhibited nearly the same dose-response behavior as expected from the same inhibition property of the G6PDH- and MDH-digoxin conjugates employed. The biotin/avidin-mediated assay, however, yielded a dose-response curve with a higher detection range than those of the EMIT-type assays: *i.e.*, 10^{-7} to 10^{-6} M for the biotin/avidin-mediated assay *vs.* 10^{-8} to 10^{-7} M for the EMIT-types. The performance of the biotin/avidin-mediated assay system is influenced by two different binding reactions: biotin/avidin and digoxin/antibody reactions. In principle, the intrinsic detection capability of the final assay is determined by the weaker binder reaction. In most biotin/avidin-mediated assays, the limit of detection is expected to be determined by the analyte binder system, since the association of biotin toward avidin is extremely strong ($K_a = 10^{-15}$ M). In the case of the digoxin assay, a limit of detection near 10^{-11} M digoxin may be possible based on the association constant of digoxin toward its monoclonal antibody ($K_a = 4 \times 10^{-11}$ M) used in this study. However, the present biotin/avidin-mediated digoxin assay was shown to exhibit a poor detectability (*i.e.*, $>10^{-7}$ M); this may be attributed to very high binder and conjugate concentrations used in the assay, even higher than those used in the EMIT-types. As can also be seen in Figure 1, all of the three assay systems yielded a relatively steep dose-response behavior over a narrow concentration range, suggesting that the assays can be used for the precise determination of digoxin. However, the detection limit achieved with these homogeneous assays is not adequate if used for serum digoxin measurements (*i.e.*, $(1.0$ to $2.5) \times 10^{-9}$ M). Therefore, a more sensitive detection scheme for the labeled digoxin or biotin is needed to develop a clinically relevant homogeneous assay for digoxin.

Figure 2 presents dose-response curves for digoxin obtained with the optimized heterogeneous methods: A) ELISA-type and B) biotin/avidin-mediated type. An ALP is known for the labeling enzyme for heterogeneous assays. In the heterogeneous assay, enzyme activities bound to a solid phase are measured, and thus, a less substituted conjugate is typically preferred for a greater enzymatic signal. Therefore, in this experiment, the less inhibited ALP-digoxin conjugate (7% by anti-digoxin antibody) and ALP-biotin conjugate (20% by avidin) were employed for the ELISA and biotin/avidin-mediated methods, respectively. For comparison purpose, the reagent concentrations were adjusted to yield dose-response curves with similar detection limits for both assay systems. As can be seen in Figure 2, the analytical signal was greater with the biotin/avidin-mediated assay than with the ELISA-type assay. In the biotin/avidin-mediated assay, ALP-biotin conjugates are bound to the avidin-digoxin conjugates already bound to the immobilized anti-digoxin anti-

body. Because of multivalency of avidin, more than one ALP-biotin molecule can bind per one avidin-digoxin molecule, resulting in signal amplification. This may explain the enhanced signal observed with the biotin/avidin-mediated assay. As can be seen in Figure 2, both heterogeneous methods achieved a much improved detection limit (*i.e.*, near 10^{-12} M digoxin) when compared to the homogeneous method. This was expected based on much lower reagent concentrations used in the heterogeneous assay protocol. The detection limits of both assay systems are thought to be sufficiently low for use in serum digoxin measurements.

In summary, several different enzyme-linked competitive binding assay methods have been examined in an attempt to develop a digoxin assay system employing anti-digoxin monoclonal antibody. The conventional EMIT- or ELISA-type assay protocols are compared with the biotin/avidin-mediated approaches. The heterogeneous types, which yield much lower detection limit than the homogeneous types, are demonstrated to be useful for serum digoxin measurements. The advantages of the biotin/avidin-mediated approaches over the conventional methods are the capabilities of using a single substrate enzyme such as ALP in the homogeneous method, and of amplifying the enzymatic signals in the heterogeneous method.

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