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Rational Environmental Management of Agrochemicals

Risk Assessment, Monitoring, and Remedial Action

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Chapter 11

Noncompetitive Fluorescent Immunoassay for the Detection of the Human Urinary Biomarker 3-Phenoxybenzoic Acid with Bench Top Immunosensor KinExA™ 3000

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A sensitive, automated, non-competitive fluorescent immunoassay was developed for quantitative analysis of 3-phenoxybenzoic acid (PBA) in human urine sampes as a putative biomarker of exposure to pyrethroid insecticides using the bench top immunoanalyzer, KinExA™ 3000 system. The key difference between the KinExA system and the enzyme-linked immunosorbent assay (ELISA) is to eliminate the PBA-antibody interaction with the coating antigen. This can be achieved by separately capturing the free PBA-antibody onto the hapten-immobilized beads when a constant amount of reaction solution in equilibrium between PBA-antibody and analyte passes through the bead-packed glass capillary column. Optimal dilution of the PBA antibody was determined when fluorescent signals of 0.5-2 were obtained and a sufficient amount of coating antigen was immobilized to ensure the capture of all free antibodies. IC_{50}s of the two KinExA methods (0.3 and 0.6 ng/mL for one- and two-step KinExA, respectively) were 3- and 6-fold better than the heterologous ELISA and were approximately 650- and 300-fold lower compared to that of the homologous ELISA (IC_{50} of 200

ng/mL). The KinExA assay was negligibly affected within tested range of pHs (5-10) and ionic strengths (1, 5, and 10X PBS). Similar urine matrix effects were observed in the two KinExA assays with a 5- to 10-fold increase in IC_{50}s when 5 and 10% of urine was contained in the reaction buffer. A high correlation (r^2 = 0.99) was observed between detected and spiked concentrations of PBA standard with average recoveries of 88-160%.

Introduction

Immunoassay has proven to be a sensitive tool to detect environmentally relevant substances such as pesticides or other toxic compounds in a variety of sample matrices (*1, 2*). The major merit of an immunoassay is the antibody-driven, high selectivity and sensitivity, which enables one to simplify sample preparation leading to rapid analysis of samples and to perform high-throughput analysis with very small sample volume. For immunoassay development, monoclonal antibodies (MAb) have some advantages over polyclonal antibodies (PAb) and are preferable to many researchers, however, PAb also gives comparable selectivity and sensitivity for immunoassay development particularly for the detection of small molecules. Enzyme-linked immunosorbent assay (ELISA) is the most widely accepted immunoassay format reported to date. ELISA methods are divided into two major formats, non-competitive and competitive. Non-competitive ELISA is mostly applicable to the detection of macromolecules such as proteins with at least two antibody binding sites. However, for the detection of small molecules, this type of non-competitive ELISA is not applicable because once the molecule binds to antibody; there is no site available for the binding of reporter molecules. So competitive ELISA is an alternative to non-competitive ELISA for small molecules. Although competitive ELISAs provide satisfactory sensitivities they are limited by the equilibrium between primary antibody and coating antigen. In other words, when antibodies have a higher affinity for the coating antigen than to the analyte during the competition step, high concentrations of analyte must be added to inhibit antibody binding to the coating antigen. This results in increased IC_{50} values. Thus, for the development of a sensitive competitive ELISA for a small molecule, it is essential to synthesize a series of competing haptens that have minor structural modifications of the immunizing hapten. These haptens must be screened to find one with lower affinity to the antibody than the target compound (*3-5*). The synthesis of competing haptens involves the use of hazardous chemicals and time consuming and laborious procedures. There have been

efforts to develop non-competitive immunoassays for small molecules. Two types of non-competitive immunoassays have been reported. The first is to use anti-idiotype antibodies generated by injecting antibody-analyte complex or recombinant antibody by recombinant DNA techniques (*6-8*). With these types of antibodies, assays can be conducted in a 96-well plate in a format similar to the sandwich-type ELISA. The second is to remove antibody interaction with the coating antigen by separating the interaction with analyte from the coating antigen so that assay sensitivity is solely dependent on affinity of antibody to target compound (*9,10*). For this, the antibody is first allowed to reach equilibrium binding with the analyte, then free antibody is separated from antibody-analyte complex. Detection can be conducted without elution or after elution of captured free antibody. Eluted antibody-analyte complex also can be used for quantification. These methods do not require synthesis of competing haptens. However, they are somewhat complicated, necessitating repeated capture and elution of antibodies for separation and quantification. To further simplify assay procedures and improve assay sensitivity, we report a very sensitive automated non-competitive flow fluorescent immunoassay for the detection of PBA in human urine samples. The PBA PAb used is one that showed a 100-fold difference in IC_{50}s between heterologous and homologous coating antigens in the ELISA.

Pyrethroids act on the axons of the nervous system by interacting with sodium channels in insects and mammals (*11*). The properties of pyrethroids such as high potency in controlling a wide spectrum of insects and low toxicity to birds and mammals have made it accepted worldwide for application in agriculture, forestry, homes, horticulture, and public health (*12-14*). Although pyrethroids are considered safe for humans, there have been concerns about long-term low level and high exposures, as well as environmental accumulation, and leaching into surface and groundwater (*15,16*). Some research has revealed that humans exposed to high levels of pyrethroids may experience suppressive effects on the immune system, endocrine disruption, lymph node and splenic damage, and carcinogenesis (*17-19*). Pyrethroids are metabolized rapidly by oxidation and hydrolytic cleavage of the ester linkage mainly to *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA) and PBA, which are further processed to conjugates such as glucuronide, glycine, taurine, and sulfate (*20,21*). Therefore, the development of a sensitive immunoassay for PBA may be a useful tool to estimate human exposure to pyrethroid insecticides. We have reported several immunoassays for the detection of pyrethroid parent compounds (*22-26*), their primary and secondary metabolites (*27-29*).

In this study, we used the Kinetic Exclusion Assay system (Sapidyne Instruments, Boise, ID), a bench top immunoanalyzer, to develop a non-competitive immunoassay for PBA detection. Limited use of the KinExA has been explored for quantitative analysis (*30-33*). In this paper, we compared two

KinExAs to heterologous and homologous ELISAs, and used the KinExA assay to analyze PBA in human urine samples.

Materials and Methods

Reagents

All reagents were of analytical grade unless specified otherwise. Bovine serum albumin (BSA), goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP), 3,3',5,5'-tetramethylbenzidine (TMB), all chemicals for buffer preparation, and PBA were from Sigma (St. Louis, MO, USA). Goat anti-rabbit IgG conjugated with Cy5 (GAR-Cy5) and Cy5 conjugation kit were purchased from Amersham Bioscience (Piscataway, NJ, USA). Desalting columns and the protein A affinity purification kit was purchased from Pierce (Rockford, IL, USA). Polymethylmethacrylate (PMMA) beads were purchased from Sapidyne (Boise, ID, USA). Buffers for ELISA and KinExA assays were normal strength PBS [1X PBS; 8 g/L of sodium chloride (NaCl), 0.2 g/L of sodium phosphate dibasic anhydrous (Na_2HPO_4), and 0.2 g/L of potassium chloride (KCl), pH 7.5], PBST (PBS containing 0.05% Tween 20), carbonate buffer [1.59 g/L sodium carbonate (Na_2CO_3), 2.93 g/L sodium hydrogen carbonate ($NaHCO_3$), pH9.6], and 0.05M citrate-acetate buffer (14.71 g/L $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 5.2). The structures of PBA and its immunizing and coating haptens are shown in Figure 2. Synthesis of the haptens for the production of PAb and assay development were described in our previous paper (*29*).

Immobilization of PBA Immunizing Hapten-BSA Conjugate to PMMA Beads

Dry PMMA beads (200 mg, 98-μm diameter) in a 1.5 mL Eppendorf tube were suspended in 1 mL of nanopure water and washed two times with PBS buffer by centrifugation and removal of the supernatant solution. Beads were then suspended with 1 mL of coating buffer, 0.1 mg of PBA hapten-BSA conjugate added, and the tubes were rolled with an end-over-end rocker for 1 h at 37 °C. After discarding the coating buffer, 1 mL of blocking buffer (1% BSA in PBS with 0.05% Tween 20) was added and the tubes rolled again for 2 h at room temperature. After the blocking buffer was discarded, 1 mL of PBS was added. Bead preparations were stored at 4 °C until use. On the day of use, the contents of two tubes were transferred into the bead reservoir of the KinExA along with 27 mL of PBST to provide a constant supply of beads into the capillary flow column.

KinExA Principles and Non-competitive Assays

KinExA is an automated flow fluorescent immunoassay system initially intended to determine the true liquid-phase equilibrium dissociation constants (K_d) and association rate constants (k_{on}) of antibodies by measuring the amount of antibodies in an equilibrium state with ligands in solution phase (*34-36*). The principle is shown in Figure 1 and has been fully described by several laboratories. (*30-36*).

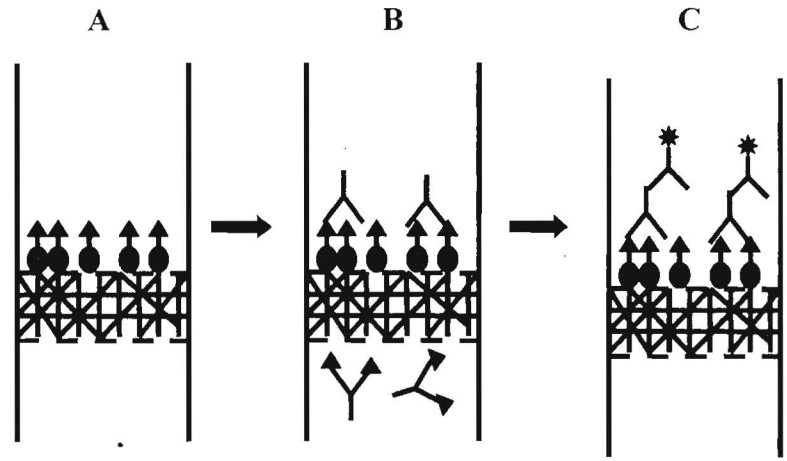

Figure 1. Schematic presentation of the KinExA principle. (A) Beads are stirred in the bead reservoir for a few seconds to homogeneously disperse hapten-immobilized beads and then transferred onto the microporous screen in the capillary flow cell. (B) Sample from one of the reaction tubes containing a fixed amount of antibody and various concentrations of analyte is injected through the rotary selector into the flow cell. While a sample passes through the cell, free antibody is captured by hapten-coated beads allowing the antibody-analyte complex to flow into the waste reservoir. (C) The secondary antibody-Cy5 is captured onto the bead by the primary PBA antibody. A fluorescent detector reads the signal of captured secondary antibody in the flow cell.

The resulting fluorescence signal is proportional to the amount of free receptor and inversely to the concentration of analyte. For the one step assay, a constant amount of Cy5-conjugated PBA antibody was mixed with an equal volume of standard solution in various concentrations. Each of 12 injection lines connected to the rotary selector valve was placed into a reaction tube and 13th line into the tube containing 10% NaOH solution. The time for appropriate bead

loading in the capillary column was determined by adjusting the time setting parameters of the KinExA software until the height of bead packing matched that of the manufacturer's measuring tool. Four hundred microliters of each sample solution was pre charged into each injection line twice to assure that there were no air bubbles in the lines. The mixtures were then incubated for 30 min at room temperature to allow them to reach equilibrium. Then, 400 µL of each reaction was passed through the bead column. After a wash with PBST, the fluorescent intensity of the captured PBA antibody was obtained. For the two-step assay, the mixture of a fixed amount of unlabeled PBA antibody and each analyte solution in various concentrations was passed through the bead column and the captured antibody was detected by adding GAR-Cy5 (1 µg/ mL). After measuring the signal, the bead column was back flushed and the column was washed successively with PBST and 10% NaOH solution. A new batch of beads was introduced into for the next round of measurement. The standard inhibition curve of twelve different concentrations of PBA was fitted using a four parameter equation with Origin 6.1 software.

Microplate ELISA

ELISAs were performed in 96-well microplates as described in Shan et al. (*27*).

Antibody labeling

Antibody labeling with Cy5 dye was performed following the manufacturer's instruction. The antibody-Cy5 conjugate was separated from free dye with a desalting column. Sodium azide was added to a final concentration of 0.02% and aliquots were stored at -20 °C.

Effects of Urine Matrices and pH

Assay tolerance to various pH and ionic strengths was estimated using PBS buffer of pH 5, 6, 7, 8, 9, and 10 and 1, 5, and 10X PBS. For an estimation of matrix effect on assay performance, PBS buffer containing 5 and 10% urine was prepared and IC_{50}s were compared to that of 0% urine.

Fortification Urine Samples with 3-PBA

PBA standard stock solution dissolved in DMSO was used for spiking urine samples. Urine samples were spiked with PBA standard and diluted with PBS buffer to render final concentrations of 0.25, 0.5, 5, 10, 15, and 20 ng/mL. The final concentration of urine was kept at 5%.

Results and Discussion

Non-Competitive Homologous KinExAs and Comparison of Sensitivities with Plate ELISAs

For the homologous non-competitive KinExA, a sufficient amount of coating antigen must be immobilized on the beads to capture all free antibodies. To test this, various dilutions of unlabeled- and Cy 5-labeled antibody were passed through the bead column containing 5 mg of beads on which 2.5 µg of PBA hapten-BSA conjugate was immobilized by adsorption (data not shown). Fluorescent signals measured in real time gradually increased as the amount of antibody increased. Unlike the typical ELISA, it is not necessary to determine the antibody dilution that saturates the immobilized coating antigen. For the two-step KinExA, the highest signal of 8.2 was observed at a dilution of 1:8000. Our previous experience with this system had shown that when the maximal fluorescent signal was set between 0.5 and 2.0, the detector of the KinExA was capable of distinguishing the differences in signals resulting from various concentrations of target compound. Thus, 1:64000 dilution of antibody was selected for the two-step assay, which gave a signal difference of 2. In contrast to the two-step assay, the fluorescent signals were significantly weaker in the one-step. This could be explained by 1) signal amplification due to multiple binding of commercial GAR-Cy5 to the captured PBA antibody in the two-step KinExA, 2) the further dilution of PBA antibody during the Cy5 conjugation and desalting procedures, or 3) probably a low molecular ratio of Cy-5 to antibody. The 1:20000 dilution of PBA antibody was selected for one-step KinExA. At the selected dilution of antibody, most of the free antibody could be captured on the beads. Microplate indirect competitive ELISAs were carried out with homologous and heterologous coating antigens and the sensitivities (IC_{50}s) were compared with those of two KinExA assays. In Figure 3, the KinExA grams show the trend in signals in the presence of various concentrations of PBA. In this case, data acquisition was not initiated until the labeled antibody was injected. Since the PBA antibody was not labeled, the two-step assay requires

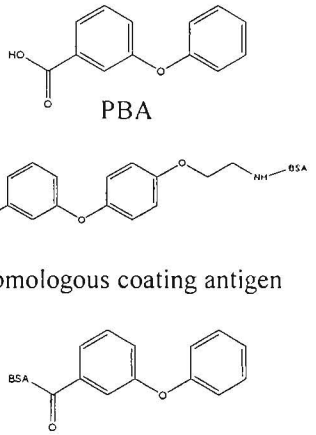

Figure 2. Structure of tet compound and coating antigens

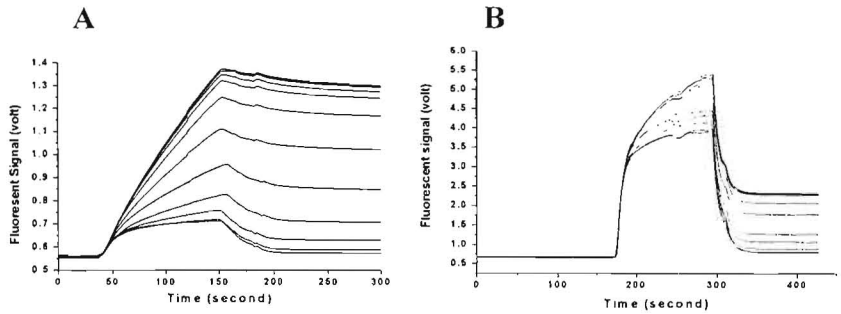

Figure 3. Example KinExAgrams in the presence of PBA in 0-2500 ng/mL. A. One-step KinExA; B. Two-step KinExA.

longer initiation times (1-170 s) than the one-step assay (1-25s) for the automatic packing of fresh beads, sample injection, and column wash. There is a substantial signal increase when labeled PBA or secondary antibody is captured on the bead (170-300 and 50-150s for two- and one-step assay, respectively) and the successive washing steps rapidly removed unbound secondary antibody so that established measurable signals were obtained. Signal intensity was inversely proportional to the concentration of PBA. Figure 4 shows representative four-parameter curves by the KinExAs and plate ELISAs. In the microplate ELISA, the IC_{50} of heterologous ELISA (2.0 ng/ml) is 100-fold lower than that of homologous ELISA (200 ng/ml), which indicates that the formation of reverse equilibrium is dominant in solution during

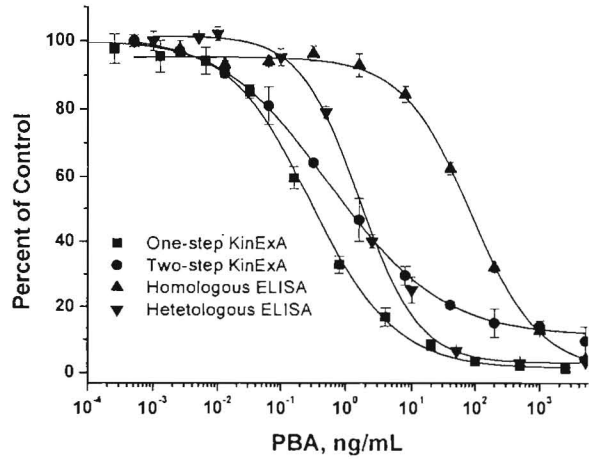

Figure 4. Four-parameter curves plotted with end-point signals of two KinExAs and ELISAs.

the competition step. The IC_{50}s of the two homologous KinExAs were 0.3 and 0.6 ng/ml for the one- and two-step assay, respectively. Compared to plate ELISAs, a significant enhancement in assay sensitivity was achieved by the homologous KinExA, approximately 650-fold improvement over the homologous ELISA and a 3- to 6-fold improvement over the heterologous ELISA. In the KinExA system, the working range was approximately 0.02-3 and 0.1-20 ng/ml for the one- and two-step assay, respectively. The one-step KinExA assay is more advantageous over the two-step assay because analysis time for each sample is significantly shortened as the addition of secondary antibody is omitted.

This minimizes photobleaching of the fluorescent dye that can cause signal variations from assay to assay. In addition, the one-step assay shows near zero background signals whereas the two-step assay always shows near 20% background signals due to nonspecific binding of secondary antibody. To generate reproducible results, it is important to keep the background signal at a constant level since the final reading signal is the difference between background and endpoint signals. Adsorption of reagents onto the column wall and insufficient washing are a principal cause for a background increase. Beginning the analysis with the most dilute samples and progressing to samples with higher concentrations can also help minimize the problem. Overnight washing of inlet lines with a solution of 0.1 N NaOH can also reduce the background. However for this assay, during assay optimization, the background increased despite the use of these measures. The problem was eliminated by introducing a 10% NaOH solution through the 13th sample injection line immediately after back-flushing the beads. It should be noted that NaOH should not be used throug the h buffer line otherwise

Matrix Effect

High tolerance of an assay to the changes in pH values and ionic strengths is desirable to accurately detect PBA in human urine samples. Antibody interaction is often affected by the physicochemical properties of the reaction solution. So the effects on the sensitivity of KinExA assay were estimated at various pH values and ionic strengths (Figure 5). For this experiment, PBS buffer with desired pH values and ionic strengths were prepared. This experiment was conducted with the one-step KinExA method. The sensitivity of the assay was negligibly affected within the range of tested pHs of the solutions. Although a slight decrease in maximal signal at pH 4 and 10 was observed, there was no significant difference in IC_{50}s indicating that the changes of pH in the range tested would not affect the accuracy. The assay was also highly tolerant to changes in ionic strength with little change in IC_{50}s and maximal signals. The effect of urine on assays was also estimated with the two KinExA methods using PBS buffer containing 5 and 10% urine (Figure 6). Effects of urine were similar

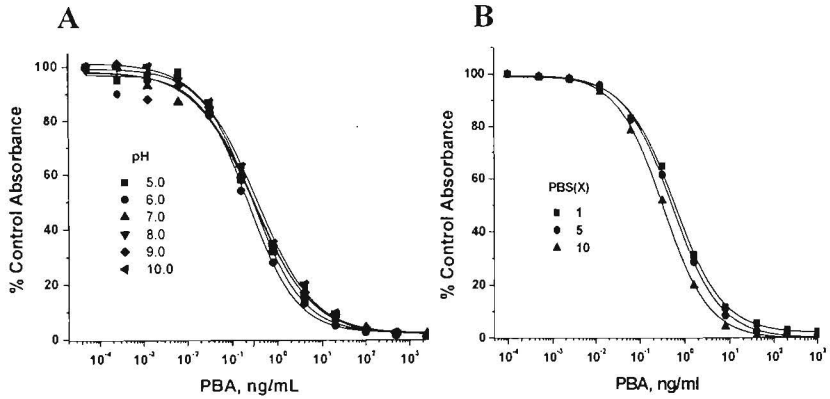

Figure 5. Effect of various pHs and ionic strengths on assay performance. A. effects of pHs; B. effects of ionic strengths. Each point represents the mean value of two replicates.

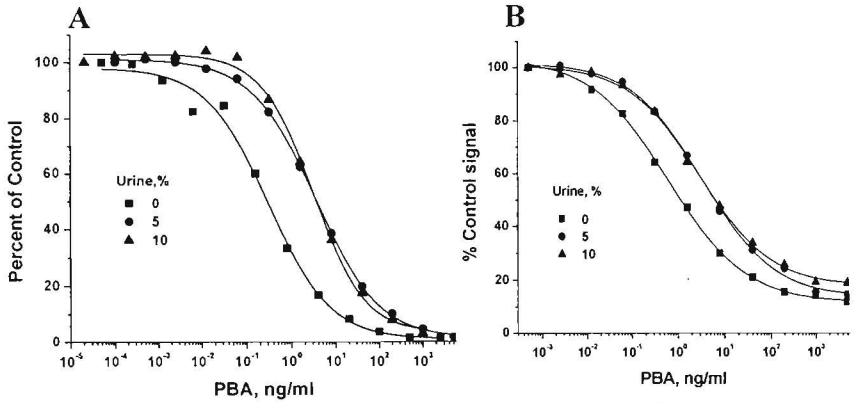

Figure 6. Effect of urin matrix on assay performance. A.effect of urine on one-step KinExA; B.effect of urine on two-step KinExA. Each point represents the mean value of two replicates

at the two urine concentrations for the two assay methods. IC_{50} values were 5- to 10-fold increased at 5 and 10% urine. However, there was negligible difference in IC_{50}s and maximal signals. The IC_{50}s were 1-3 ng/ml indicating that use of the same percent of control urine for the preparation of standard curves may allow us to accurately detect unknown samples using these dilutions.

Assay validation

The assay validation was performed with the one-step KinExA method. Since the stability of conjugated Cy5 was not affected by incubation with sample during equilibrium, the one-step method was the first choice for this experiment. Recovery tests were conducted with a human urine sample which was spiked with PBA standard solution at 0, 0.5, 2.5, 5, 10, 15, and 20 ng/mL. Linear regression analysis of the results showed a good correlation between spiked and detected levels (Figure7). Average recoveries were ranged between 88 and 160% of the spiked values with r^2 of 0.99. These results demonstrated that this assay is able to detect the pyrethroid metabolite at trace levels in urine samples. Biomonitoring of human urine samples conducted by Centers for Desease Control and Prevention during 2001-2002 revealed that measurable amount of PBA (0.3 ng/mL) was found in people of age 6 and older. It should be noted that when the set of measurements was conducted four times successively with no change of reagents (equivalent to 48 samples), we observed significant false positive results from the 3rd measurement indicating that photobleaching of fluorescent dye occurs during prolonged use. One way to avoid this phenomenon is to use freshly prepared fluorescent-labeled antibody solution after every one or two sets of measurement. In addition, if this system is to be used for the analysis of many samples, more intensive efforts should be put into an estimation of urine matrix effect because urine samples of individual persons may have different matrix effects that would result in false positive or negative detection.

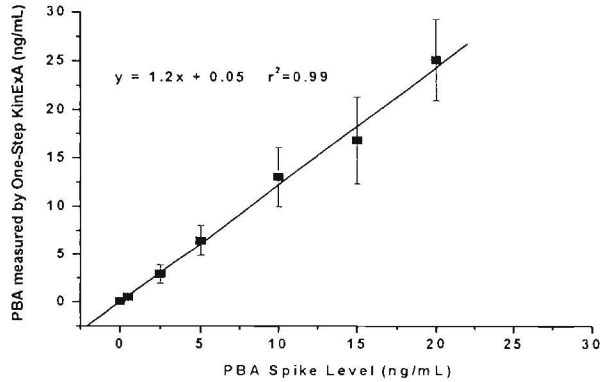

Figure 7. Relationship between spiked PBA in urine and measured by one-step KinExA.

Conclusion

Development of sensitive one- and two-step noncompetitive assays with the bench top immunoanalyzer, KinExA 3000 system for the detection of a urinary biomarker, PBA in human urine samples is described in this study. Unlike the typical ELISA method, the ability of the KinExA system to separately capture free PBA antibody from the PBA-antibody complex while the sample passed through the bead column greatly improved assay sensitivity. This homologous type of KinExA method eliminates the need of laborious synthesis of competing haptens. It also eliminates the optimization of coating antigen concentration once a sufficient amount of homologous hapten is immobilized on the solid support.

Optimization of coating antigen is frequently carried out by a two-dimensional checkerboard titration method in ELISA. For the proof of the concept, we used a PBA PAb. In our previous study, this antibody showed approximately 100-fold difference in IC_{50}s between heterologous and homologous antigens (*27*). The comparison of sensitivities between KinExA methods and ELISAs showed that two homologous KinExA methods had several-fold better IC_{50} values than heterologous ELISAs. The IC_{50} value of the one-step KinExA was a 650-fold improvement over the homologous ELISA. In addition to enhanced sensitivity, another advantage of this system is the high degree of PC-based automation that allows ones to easily set parameters to modulate the injection amount and flow rate of buffer or reagent solution for the best sensitivity eliminating the manual multi-step washings and incubations of ELISA. Once the system is optimized for a target compound, the KinExA consistently performs so that many samples could be consecutively analyzed providing real time results without constructing additional standard curves. One sample could be analyzed within a few minutes. In the case that many samples are to be analyzed with the KinExA, although the one-step assay is fast and more sensitive, the two-step assay is advantageous. A freshly prepared GAR-Cy5 solution can be easily replaced by simply changing out the reservoir with a new one, which prevents the fluorescent dye from photobleaching during the prolonged incubation.

The shape of the KinExAgram is similar to that of a surface plasmon resonance (SPR) assay (*37*). However, the main difference is that the KinExA system requires fluorescent labeling of the reporter molecule whereas the SPR system utilizes a sensor chip-based label-free detection system. The SPR system is among the various types of biosensors that have been reported for the detection of environmental substances (*1, 38*). The main principal of a biosensor is to immobilize recognition elements (e.g., enzyme, antibody, or receptor) on the surface of sensor material connected to the signal transducer. For consecutive analysis, sensor material must be regenerated after one measurement or replaced with new one, making this approach less attractive for high throughput analysis.

KinExA uses relatively cheap polymers for the immobilization of recognition elements (e.g., antibody or coating antigen). KinExA's fluidic system back-flushes the used material after one measurement is finished and accurately introduces controlled amounts of the new batch of beads. For example, the 200 mg of PMMA beads used in this study allowed 36 consecutive sample analyses. In addition, covalent immobilization of coating antigen is unnecessary for this system. PMMA beads could be coated and blocked within 2 h. In this study, it was important to immobilize on the beads, a sufficient amount of coating antigen with high affinity for antibody to capture all free antibody when the mixture of antibody and analyte pass through the bead bed.

The assay was tolerant to various pH values and ionic strengths. Urine matrix effects were somewhat observed in PBS buffer containing 5 and 10% urine. However, except that the IC_{50} values were slightly increased, good curve shapes and reproducible IC_{50} values of 1-3 ng/ml were obtained. This indicates that when the same amount of control urine is added for the preparation of the standard curve, unknown urine samples could be analyzed. Further optimization may be necessary to meet the relevant concentration range in the prescence of urine. A good correlation was observed between the recoveries by KinExA and spiked level.

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