Published in final edited form as:

J Agric Food Chem. 2018 October 31; 66(43): 11284–11290. doi:10.1021/acs.jafc.8b04521.

Development of a Nanobody-Alkaline Phosphatase Fusion Protein and Its Application in a Highly Sensitive Direct Competitive Fluorescence Enzyme Immunoassay for Detection of 3-Phenoxybenzoic Acid in Urine

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Abstract

3-Phenoxybenzoic acid (3-PBA), **1**(Figure 1) is a human urinary metabolite of many pyrethroid insecticides and can be used as a biomarker to monitor human exposure to these pesticides. A rapid and sensitive direct competitive fluorescence enzyme immunoassay (dc-FEIA) for 3-PBA based on a nanobody (Nb)-alkaline phosphatase (AP) fusion protein was developed. The anti-3-PBA Nb-AP fusion protein was expressed and purified. The 50% inhibitory concentration (IC₅₀) and the detection limit of the dc-FEIA were 0.082 and 0.011 ng/mL, respectively, with a linear range of 0.015–0.447 ng/mL. The IC₅₀ of the one-step dc-FEIA was improved by nearly ten times compared with that of the one-step and three-step dc-ELISA. This assay was also compared with LC-MS for detecting the spiked urine samples, and the results indicated the reliability of Nb-AP fusion protein-based dc-FEIA for monitoring 3-PBA in urine.

Graphical Abstract

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Supporting Information

The synthetic gene for Nb-AP fusion protein, optimized parameters of LC and MS and structure of immunization antigen and coating antigen were supplied in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

3-PBA; nanobody-alkaline phosphatase fusion protein; one-step competitive fluorescence enzyme immunoassay

Introduction

Pyrethroids, one of the most widely used insecticide classes in the world, are mainly applied in agriculture, forestry and horticulture for controlling insect pests.¹ Also, pyrethroids can be used as insecticides in indoor environments and are found in medication used for treating for scabies and topical louse infestations.² The pyrethroids are among the safest insecticides marketed. However, because of the numerous applications and increasing worldwide use of pyrethroids, a variety of people are exposed to pyrethroids at levels that could be harmful to humans and particularly to vulnerable subpopulations such as children.³ For example, the potential effects include endocrine disruption,⁴ DNA damage in human sperm,⁵ primary ovarian insufficiency,⁶ liver injury,⁷ sensory alterations,⁸ and others.^{9, 10} Therefore, it is important to develop a rapid, sensitive, and efficient analytical method for environmental monitoring and assessment of human exposure to pyrethroids and to aid in proper use of these compounds. Most pyrethroids contain a phenoxybenzyl alcohol or cyanohydrin (Figure 1) and both of these esters can be hydrolyzed and converted to the corresponding acid. Thus, 3-PBA is a human urinary metabolite or breakdown product of many pyrethroid insecticides.¹¹ The concentration of 3-PBA in urine provides information about a person's recent exposure to pyrethroid insecticides. Higher urine concentrations generally indicate more exposure, therefore, 3-PBA can be used as a biomarker to monitor human exposure to pyrethroid insecticides.^{12, 13}

At present, the detection methods for 3-PBA include supercritical fluid chromatography,¹⁴ gas chromatography,¹⁵ high-performance liquid chromatography,¹⁶ immunoassays,^{17, 18} and optical sensing.¹⁹ Among the above methods, immunoassay stands out because of its unique ability to routinely handle many samples and because it also does not require time-

consuming procedures and sophisticated equipment when compared with the instrumental methods. Most of the previously reported immunoassays for 3-PBA are based on a monoclonal antibody or a polyclonal antibody^{11, 20} and secondary or tertiary antibodies are required in the process. Due to the advances in recombinant DNA technology, construction of an antibody-AP fusion has been proposed and used for simple and rapid immunoassay analysis.²¹ One study on the one-step detection of O,O-diethyl organophosphorus pesticides based on the recombinant fusion protein of single chain variable fragment (scFv) and AP has been reported.²² The single variable domain of a heavy chain (VHH) antibodies, also called as nanobodies (Nbs), with properties such as small size, high solubility and stability, has become as an attractive alternative to conventional scFv. It has been reported that the fusions of AP to Nbs were applied in the detection of proteins²³ as well as small molecules, such as mycotoxins²⁴ and tetrabromobisphenol A.²⁵ Until now, there have been few reports on the application of Nb-AP fusions for the detection of pesticides or their metabolites. Recently, many fluorescent phosphatase substrates have been developed and commercialized. Most of those new fluorescent substrates are highly sensitive, maintaining a low fluorescence signal until enzymatically acted upon and even yielding detection of AP as low as 0.1 attomole.²⁶ Based on the highly sensitive substrate, the fluorescence assay allowed for more sensitive detection than the colorimetric assay.²⁷ Hence, Nb-AP fusions could be a powerful reagent for trace detection of pesticides or their metabolites.

In our previous work, a phage VHH library was constructed based on an immunized alpaca, and seven VHH clones were selected by competitive binding with 3-PBA.²⁸ In this study, the VHH which showed the highest sensitivity was used to construct the Nb-AP fusion. The Nb gene was cloned into the expression vector peca45, which contained the AP gene²⁹ to produce the Nb-AP fusion protein. After the characterization by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the purified fusion protein was used to develop a rapid, simple, and sensitive direct competitive fluorescence enzyme immunoassay for detection of 3-PBA in urine.

Materials and Methods

Chemicals and Reagents.

T4 DNA ligase and restriction enzyme *SfiI* were obtained from New England Biolabs, Inc. (Beverly, MA). Phusion High-Fidelity DNA Polymerase, bacterial protein extraction reagent (B-PER), HisPur Ni-NTA resin, NuPAGE Bis-Tris Gel (the precast polyacrylamide gels designed to give optimal separation for a wide range of molecular weight proteins during gel electrophoresis) and chemically competent cells of *E. coli* BL21 (DE3) pLysS were from Thermo Fisher Scientific (Rockford, IL). Standards (3-phenoxybenzoic acid, **1**, and its analogs, 3-phenoxybenzyl aldehyde and 3-phenoxybenzyl alcohol; permethrin, **2**; cypermethrin, **3**; deltamethrin, **4**; fenpropathrin, **5**; and phenothrin, **6**) (Figure 1)), isopropyl- β -D-thiogalactopyranoside (IPTG), and *p*-nitrophenyl phosphate (pNPP) substrate were from Sigma (St. Louis, MO). The AttoPhos AP fluorescent substrate system was purchased from Roche (Pleasanton, CA). The vector peca 45 containing AP gene was a generous gift from Dr. Jinny L. Liu and Dr. Ellen R. Goldman (Naval Research Laboratory, Center for

Bio/Molecular Science and Engineering, Washington, DC). The AP-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Inc. (West Grove, PA).

Construction of the Recombinant Plasmid, Expression and Identification of Nb-AP Fusion Protein.

The recombinant plasmid encoding the Nb-AP fusion protein with a 6X His tag at its C-terminal end was constructed. Briefly, primers AP-F and AP-R were used to amplify the Nb gene, followed digestion with *Sfi*I restriction enzyme (forward primer: GAG GAG GAG GTG GCC CAG CCG GCC CAG GTG CAG CTC GTG GAG TCT GGG GGA; reverse primer: GAG GAG GAG CTG GCC CCC GAG GCC GCG TCT TGT GGT TTT GGT GTC TTG GG). The Nb fragment was then ligated into the similarly digested expression vector pcan45 containing AP gene at a 10:1 molar ratio using T4 DNA ligase, followed by transforming the ligation products into the chemically competent cells of the *E. coli* strain BL21(DE3) pLysS by heat shock (42 °C, 30 s). The transformed bacteria were seeded on super broth (SB) agar plates containing 50 µg/mL ampicillin (Amp), and positive clones were picked for plasmid extraction and DNA sequencing (Division of Biological Sciences, Automated DNA Sequencing Facility, University of California, Davis).

The colony containing the recombinant plasmid was cultured in 10 mL SB medium with 50 µg/mL Amp at 37 °C overnight. Then, the overnight culture was inoculated 1: 100 (v/v) in 1 L of SB medium containing 50 µg/mL Amp and incubated at 37 °C until the OD 600 reached approximately 0.6. The culture was then induced with 0.1 mM IPTG at 25 °C and was shaken at 250 rpm overnight. The bacterial cells were collected by centrifugation at 10 000 g for 20 min, and the soluble fusion protein was extracted by B-PER method according to the manufacturer's instructions.

Purification of the Anti-3-PBA Nb-AP Fusion Protein.

The extracted Nb-AP fusion protein, which contains a 6X His tag, was first filtered through a 0.22 µm sterile filter (MilliporeSigma, Temecula, CA), followed by loading onto a high-capacity nickel immobilized metal ion affinity chromatography (IMAC) resin column for purification. After being washed with six resin-bed volumes of wash buffer (10 mM PBS containing 25 mM imidazole, pH 7.4), the Nb-AP fusion protein was eluted with 6 mL of elution buffer (10 mM PBS containing 100 mM imidazole, pH 7.4). After dialysis with 10 mM PBS (pH 7.4) at 4 °C for 72 h, the obtained Nb-AP fusion protein was stored at -20 °C until use. The purity of the resulting Nb-AP fusion protein was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using the NuPAGE Bis-Tris Gels according to the manufacturer's instructions.

The AP enzyme activity of the Nb-AP fusion protein. ²⁷

Colorimetric analysis is the most common method to measure the AP enzyme activity. In order to compare the sensitivity of colorimetric analysis with the fluorometric analysis, here, we measured the AP enzyme activity using the two analyses according to the reported method. ²⁷ Briefly, serially diluted Nb-AP fusion protein was added into 96-well microplate, followed by addition of substrates. The mixture was incubated for 30 min and 15 min at room temperature for colorimetric analysis and fluorometric analysis, respectively. The

reaction for colorimetric analysis was stopped with 3 M NaOH and then the absorbance was measured at 405 nm. While, the fluorescence was measured at 440 nm excitation wavelength and 550 nm emission wavelength. Generally, the colorimetric analysis used the ordinary 96-well microplate while the fluorometric analysis using the black opaque 96-well microplate. The substrates for colorimetric analysis and fluorometric analysis were *p*-nitrophenyl phosphate and 2'-(2-benzothiazoyl)-6'-hydroxybenzothiazole phosphate, respectively.

One-step competitive immunoassay for 3-PBA Based on Nb-AP Fusion Protein.

Colorimetric Enzyme Immunoassay—3-Phenoxybenzoic acid/bovine serum albumin conjugate in PBS (10 mM, pH 7.4) (400 ng/mL, 100 μ L/well) was coated in microplates overnight at 4 °C and blocked with 200 μ L/well 3% skim milk in PBS (10 mM, pH 7.4) for 1 h at room temperature. After washing with PBST (10 mM, pH 7.4, 0.5% Tween 20) three times, 500 ng/mL of Nb-AP (50 μ L/well) and various concentrations of 3-PBA standard (0.01, 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100 ng/mL, 50 μ L/well), both diluted with PBS (10 mM, pH 7.4) were added; the mixture in the plate was incubated at 25 °C for 1 h. After washing five times with PBST, the enzyme activity in the wells was determined with the colorimetric assay described above, and standard curve was established by plotting the value of B/B₀ (%) against the 3-PBA concentration, where B is the absorbance in the presence of 3-PBA and B₀ is the absorbance in their absence.

For comparison, three-step competitive ELISA (colorimetric enzyme immunoassay) for the parental anti-3-PBA VHH fused with C-terminal hemagglutinin (HA)-tag was also performed. Microwells were coated with 100 μ L/well 50 ng/mL 1/BSA conjugate and then incubated overnight at 4 °C. After blocking with 3% skim milk (200 μ L/well) in 10 mM PBS for 1 h at room temperature and then washing with PBST, each serial concentration of 3-PBA (0.01, 0.1, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100 ng/mL, 50 μ L/well) equally with 450 ng/mL VHH was added to the wells and incubated at 25 °C for 1 h. Following washing five times with PBST, 1:5000 dilution of anti-HA-tag mouse monoclonal antibody (100 μ L) was incubated in the wells at 25 °C for 30 min. After washing five times with PBST, in order to use the same substrate with the one-step competitive immunoassay (colorimetric enzyme immunoassay), 1:5000 dilution of AP-conjugated goat anti-mouse IgG (100 μ L) was incubated in the wells at 25 °C for 30 min. Finally, AP substrate pNPP (150 μ L) was added to the wells for 15 min and the reaction was stopped with 3M NaOH. The optical density at 405 nm was determined on a microplate reader.

Fluorescence Enzyme Immunoassay—For this assay, a black opaque 96-well microplate was incubated with 100 μ L/well of 25 ng/mL 1/BSA conjugate in PBS (10 mM, pH 7.4) at 4 °C overnight. After blocked with 3% skim milk in 10 mM PBS (200 μ L/well) at room temperature for 1 h, the plate was washed three times with PBST. Subsequently, 50 μ L/well of 20 ng/mL Nb-AP fusion protein diluted in 10 mM PBS was added and incubated with 50 μ L/well of serial concentrations of 3-PBA standards (0, 0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 ng/mL in 10 mM PBS) at 25 °C for 1 h. After five washings with PBST, the plate was incubated with 100 μ L/well of the 2'-(2-benzothiazoyl)-6'-hydroxybenzothiazole phosphate at room temperature for 15 min. The fluorescent signal was measured as described above, and standard curve was established by plotting the value of

F/F_0 (%) against the 3-PBA concentration, where F is the fluorescence intensity in the presence of 3-PBA and F_0 is the fluorescence intensity in its absence.

Analysis and Validation of Spiked Urine Sample based on Direct Competitive Fluorescence Enzyme Immunoassay (dc-FEIA) and LC-MS.

Urine samples used for the spike recovery study were collected from person with no known exposure to pyrethroid insecticides and were confirmed to be free of 3-PBA by LC-MS. Urine samples fortified with 3-PBA (1, 2, and 4 ng/mL) were diluted with 10 mM PBS to reach a final percentage of 5% urine/PBS (v/v). After complete mixing, the diluted samples were subjected to the one-step fluorescence enzyme immunoassay. LC-MS analysis was performed on Waters Acquity UPLC system, coupled to Xevo TQ-S Triple Quadrupole LC-MS. The above spiked urine samples (50 μ L with the 3-PBA of 1, 2, and 4 ng/mL) were mixed with 50 μ L 200 nM 12-(3-cyclohexyl-ureido)-dodecanoic acid in methanol, which was used as an internal standard to account for ion suppression, before detection. All data were acquired and processed using Masslynx 4.1 software with TargetLynx.

Results and Discussion

Expression, Purification, and Characterization of the Nb-AP Fusion Protein.

To create a convenient one-step detection for 3-PBA, the plasmid pecan45-Nb-AP was constructed by inserting the Nb gene into the expression vector pecan45 containing AP gene. The positive recombinant plasmid was confirmed by colony PCR and DNA sequencing. Resulting plasmid was then transformed into *E. coli* strain BL21(DE3) pLysS for the expression of the soluble fusion protein. The periplasmic protein was extracted by the B-PER reagent and was purified by the Ni-NTA affinity column. Protein size and integrity were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 2). The gels of selected purified Nb-AP fusion protein showed the expected band of approximately 65 kDa for the 1:1 fusion of Nb and AP.

AP Enzyme Activity and Anti-3-PBA Reactivity of Nb-AP Fusion Protein.

The AP enzyme activity of Nb-AP fusion protein was evaluated with colorimetric and fluorometric analysis. As shown in Figure 3, the signal intensity decreased as the amount of Nb-AP fusion protein decreased in both the fluorometric assay and colorimetric assay. The limit of detection (signal to noise > 3) for AP enzyme activity in the fluorometric assays (dilution factor 4000 times) was approximately 20 times lower than that in the colorimetric assay (dilution factor 200 times), indicating the higher sensitivity of fluorometric assay compared with colorimetric assay.

To evaluate the anti-3-PBA reactivity of Nb-AP fusion protein and compare its sensitivity with the parental VHH, one-step and three-step direct competitive ELISA were carried out. The concentrations of coating antigen and antibody were determined by checkerboard titration. As shown in Figure 4, it is obvious that the binding between the antibody and coating antigen can be inhibited by free 3-PBA, and the 50% inhibition concentrations are 0.811 ng/mL and 0.740 ng/mL for one-step ELISA and three-step ELISA, respectively, showing the similar sensitivity in 3-PBA detection. This result indicated that Nb-AP fusion

protein had similar binding properties to the parental VHH in the immunoassay, while the one-step immunoassay based on Nb-AP fusion protein significantly reduced the assay time to 1 h compared with three-step ELISA that require more than 3 h.

FEIA for 3-PBA Using Nb-AP Fusion Protein as the Probe

The fact that fluorometric assay was 20 times more sensitive than colorimetric assay for the detection of AP enzyme activity (Figure 3) indicated the higher sensitivity in FEIA could be attained compared to the conventional immunoassay. Therefore, the direct competitive FEIA based on Nb-AP was developed and the conditions for best performance of dc-FEIA were investigated in this study. Since many assay parameters can influence immunoreactions,^{30, 31} optimization of the concentration of coating antigen and antibody, ionic strength and pH value were important to increase the sensitivity of 3-PBA detection. The working concentrations of coating antigen 1/BSA conjugate (25 µg/mL) and Nb-AP fusion protein (20 ng/mL) were first determined by a checkerboard titration. To evaluate the effect of the ionic strength on the assay performance four different concentrations of PBS (5, 10, 25, and 50 mM) were tested. As can be seen from Figure 5A, the fluorescence intensity decreased as the ionic strength increased. Although the IC₅₀ were not significantly different with different concentrations of PBS buffer, the lowest IC₅₀ of 0.076 ng/mL was observed at 10 mM PBS. The influence of the pH on FEIA was evaluated in the range pH 4.0–11.0 (Figure 5B). The FEIA was most influenced at pH 6.0 and 11.0, with the IC₅₀ being 0.411 and 0.480 ng/mL, respectively. The maximum relative fluorescence unit (RFU_{max}) was observed abnormally at very low and high pH, and the performance of Nb-AP did not exhibit best activity. The best assay performance was obtained at pH 7.4, while, pH values between 7.4 and 9.0 are equally suitable for the assay.

A direct competitive Nb-AP fusion protein-based FEIA standard curve was established using the optimal conditions (Figure 6). The standard curve exhibited a good correlation coefficient of 0.994 with the limit of detection of 0.011 ng/mL. The assay has a linear range of 0.015–0.447 ng/mL and an IC₅₀ of 0.082 ng/mL. The IC₅₀ of the one-step competitive FEIA was improved by almost ten times compared to the one-step competitive colorimetric ELISA (IC₅₀ = 0.811 ng/mL) and the three-step competitive colorimetric ELISA (IC₅₀ = 0.740 ng/mL).

Cross-Reactivity.

The specificity of the assay was tested for Nb-AP fusion protein, using two 3-PBA analogues and five parent pyrethroids (Table 1). The cross-reactivity was determined using the formula:

$$[(IC_{50} \text{ of 3-PBA})/(IC_{50} \text{ of cross-reacting compounds})] \times 100.$$

As shown in Table 1, 3-phenoxybenzyl aldehyde had a highest cross reactivity of 22.6%. The result was consistent with the cross-reactivity pattern for the parental VHH,²⁸ indicating that Nb-AP fusion preserved recognition features of the original VHH after fusion manipulation. Negligible cross reactivity with other 3-PBA analogues and parent pyrethroids

demonstrated good selectivity of Nb-AP fusion protein in dc-FEIA effectively guaranteed the detection of 3-PBA.

Matrix Effect.

Matrix effects are common challenges for immunoassays, because they can not only cause false positive and lower sensitivity but also can reduce the specificity of the assay.³² The matrix effects can be reduced in many ways. Sample cleanup procedures such as solid-phase extraction are very useful for the removal of interferences.^{33–35} Otherwise, dilution of the sample with assay buffer is another common method to reduce the matrix effects on immunoassay.³⁶ Since the main intended use of the developed FEIA method is for the evaluation of human/animal exposure to pyrethroids by detecting their common urinary metabolite 3-PBA, urine was selected for the matrix effect evaluation. Urine samples were collected from healthy volunteers and confirmed to be free of 3-PBA by LC-MS (LOD=0.01 ng/mL, LOQ=0.034 ng/mL) analysis. Undiluted urine, urine diluted 5-, 10-, 20-, 40- and 50-fold in the assay buffer and assay buffer were used to prepare serial concentrations of 3-PBA standards for the dc-FEIA. As shown in Figure 7, undiluted urine sample and the 5-fold diluted urine sample showed low fluorescence and did not have an IC_{50} value, which indicated that the high concentration of urine had a significant influence on the performance of Nb-AP fusion. No significant reduction of maximum fluorescence intensity was observed among the 10-, 20-, 40-, 50-fold diluted urine samples and assay buffer, but the sensitivity was slightly affected in the 10-fold diluted urine sample, indicating the Nb-AP fusion protein was resistant to matrix effects caused by low concentrations of urine. Considering variation among different urine samples, a 20-fold dilution was chosen as an optimal dilution for the developed assay. In general, dilution with assay buffer is a simple and effective method for reduction/elimination of the matrix effect in immunoassay, but the disadvantage of dilution is that it results in lower sensitivity. In our study, even with a 20-fold dilution factor, the final sensitivity of dc-FEIA for urine sample was maintained in the acceptable range due to the high sensitivity of fluorometric analysis format.

Validation Study.

To evaluate the effectiveness of the assay for 3-PBA analysis, the Nb-AP fusion protein-based FEIA was performed to detect 3-PBA in the spiked urine samples. Before the spike and recovery study, all urine samples were confirmed to be free of 3-PBA by LC-MS. Urine samples spiked with three different concentrations of 3-PBA (1, 2 and 4 ng/mL) were prepared for analysis. As shown in Table 2, the average recoveries ranged from 84% to 109% for the FEIA assay, with the coefficient of variation ranging from 0.046 to 0.136, and the average recoveries ranging from 102% to 119% for the LC-MS assay, with the coefficient of variation ranging from 0.076 to 0.154. These results indicate the reliability, accuracy and reproducibility of the Nb-AP fusion protein-based dc-FEIA for 3-PBA detection in urine samples.

In this study, a rapid and sensitive one-step competitive fluorometric enzyme immunoassay for detecting 3-PBA in urine samples was successfully developed based on a novel Nb-AP fusion protein. The fluorometric detection exhibited remarkable advantages in terms of sensitivity compared to conventional colorimetric techniques. The IC_{50} of the one-step

competitive fluorometric enzyme immunoassay was nearly ten times higher compared with that of the one-step and three-step competitive colorimetric enzyme immunoassays. The high sensitivity of dc-FEIA compensated for the loss of sensitivity during the dilution of urine sample. In addition, one-step detection format based on Nb-AP fusion protein speeds up the assay procedure because no secondary or tertiary antibodies are required for the one-step dc-FEIA. With these advantages, all the procedure for 3-PBA detection could be accomplished in 2 h from sample dilution to data analysis. These results indicate that the construction of Nb-AP fusions can be considered as an attractive and powerful reagent for immunoassay analysis and the development of one-step competitive fluorometric enzyme immunoassay can be a simple and rapid analytical tool for quantitative determinations of pesticides or their metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

This work was financially supported by the National Institute of Environmental Health Science Superfund Research Program (P42ES004699), the National Nature Science Foundation of China (No. 31471786) and the National Academy of Sciences, NAS Subaward (No. 2000009144).

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Figure 1.
Structure of 3-phenoxybenzoic acid, **1**, and pyrethroids containing a phenoxybenzyl group, **2–6**.

Figure 2.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of expression of the Nb-AP fusion protein. Blots were stained with SYPRO Ruby protein gel stain. Key: lane M, PageRuler unstained protein ladder and spectrum multicolor broad-range protein ladder. lane 1, the whole cell extract of Nb-AP under induced conditions; lane 2, the wash buffer from the Ni-NTA purification; lane 3, Nb-AP fusion protein following the Ni-NTA column.

Figure 3. Dose-response curves for AP enzyme activity of the purified Nb-AP fusion protein in colorimetric (●) and fluorometric (■) assays. $n = 3$.

Figure 4.
Standard curves for one-step and three-step colorimetric immunoassays (ELISA).

Figure 5. Effects of (A) ionic strength and (B) pH on the performance of dc-FEIA for 3-PBA. n = 3.

Figure 6.

Standard competitive binding curve of Nb-AP fusion protein-based dc-FEIA for 3-PBA under optimized parameters. $C_{cAg} = 25$ ng/mL. $C_{Nb-AP} = 20$ ng/mL. $n = 3$.

Figure 7.

The effect of urine matrix on the performance of Nb-AP fusion protein-based dc-FEIA. n = 3.

Table 1.

Cross Reactivity of Nb-AP Fusion Protein with 3-PBA Structural Analogues and Five Parent Pyrethroids

Analytes	Cross-reactivity (%)
3-PBA	100
3-Phenoxybenzyl aldehyde	22.6
3-Phenoxybenzyl alcohol	<0.01
Permethrin	<0.01
Cypermethrin	<0.01
Deltamethrin	<0.01
Fenpropathrin	<0.01
Phenothrin	<0.01

Table 2.

Recoveries of 3-PBA from Spiked Urine Samples

3-PBA added(ng/mL)	FEIA(ng/mL)	Average recovery(%)	CV(%)	LC-MS (ng/mL)	Average recovery(%)	CV(%)
1	1.04±0.05	104	4.59	1.11±0.17	102	15.37
2	1.92±0.09	96	4.87	2.47±0.19	119	7.56
4	4.35±0.41	109	9.46	4.63±0.47	114	10.08

Note: Each assay was performed in triplicate on the same day.