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Preparation of Polyclonal Antibodies to a Derivative of 1-Aminohydantoin (AHD) and Development of an Indirect Competitive ELISA for the Detection of Nitrofurantoin Residue in Water

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Nitrofurans are used widely to treat animal diseases and were identified as the major compounds in many worldwide drug residue violations. To develop a rapid and convenient detection method to measure the residue of nitrofurantoin, we designed an immunogen and prepared a polyclonal antibody to develop an immunoassay in this study. The antibodies obtained were characterized by an indirect cELISA method and showed excellent specificity and sensitivity with IC_{50} of 3.2 ppb and no cross-reaction with most related species and compounds. Considering that nitrofurans often are used illegally to feed animals through drinking water, we measured the residue of nitrofurantoin in water spiked by the drug. The recovery rates are in the ranges of 88−103% for interassay and 90−103% for intra-assay. The CVs are in the ranges of 3.1−11.4% for interassay and 2.7−6.2% for intra-assay. The detection limit was determined to be 0.2 ppb. The immunoassay developed in this study is suitable to be used as a screening method to detect residues of nitrofurantoin in drinking water for animals.

KEYWORDS: Nitrofuran; nitrofurantoin; drug residue; antibody; immunoassay

INTRODUCTION

Nitrofurans are a group of synthetic broad-spectrum antibiotics that are frequently employed in animal production for their excellent antibacterial and pharmacokinetic properties to treat and prevent gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. They are also used as growth promoters in pig, poultry, and fish production. It was found by long-term studies with experimental animals that the parent drugs and their metabolites showed carcinogenic and mutagenic characteristics (*1*, *2*). Therefore, nitrofurans have been prohibited to treat animals used for food production in many countries in the world. Among the four most commonly used nitrofurans, furaltadone, nitrofurantoin, and nitrofurazone were banned from use in food animal production in the European Union (EU) in 1993, and the use of furazolidone was banned in 1995 (see **Figure 1** for their structures) (*3*). In China, nitrofurans belong to a group of animal drugs that are strictly prohibited from use in animal husbandry (*4*). However, nitrofurans are still used illegally in many countries and regions due to their effectiveness and lower cost to use. In 2006, there were several food safety accidents related to illegal drug residues in China, and nitrofurans and their metabolites were found in food animal products in these events. Realizing the seriousness of the threat resulting from nitrofuran residues in food, a project called FOODBRAND was launched in the EU in January 2000 to study detection methods for these drugs. So far, the project has made progress, and research related to the preparation of antibodies and the development of immunoassays to detect the residue of 3-amino-2-oxazolidinone (AOZ), a metabolite of furazolidone, has been reported (*5*−*8*). However, we have not found any reports of research to develop immunoassays to detect residues of the other three nitrofurans and their metabolites yet.

To detect residues of a drug in food and food products, it is a common practice to combine low-cost, high-volume screening methods with high-cost but low-volume confirmatory methods to ensure efficient and cost-effective use of resources. The standard confirmation methods to detect the residues of nitrofurans and their metabolites are instrumental methods including HPLC, LC-MS, and LC-MS/MS (*9*, *10*). The triple-quadrupole LC-MS/MS is, especially, an accurate means to detect drug residues used in many reference laboratories in the world. The enzyme-linked immunosorbent assay (ELISA), studied in this research, is a well-accepted convenient and low-cost means used worldwide as screening method to detect drug residues.

The analytical methods studied so far to detect nitrofurans residues in food and food products focus on detecting their tissue-bound metabolites AOZ, 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ, a metabolite of furaltadone), 1-aminohydrantoin (AHD, a metabolite of nitrofurantoin), and

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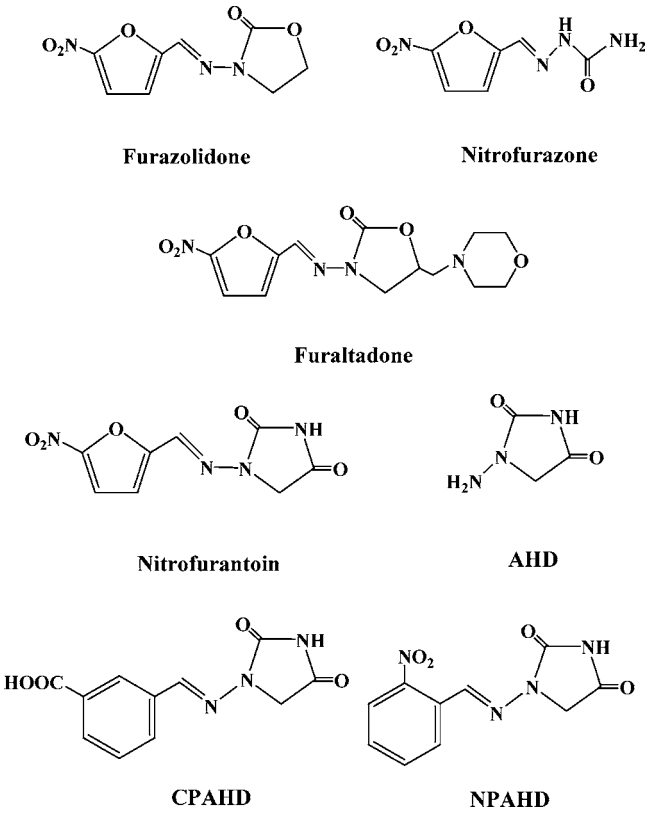

Figure 1. Chemical structures of furazolidone, nitrofurazon, furaltadone, nitrofurantoin, AHD, CPAHD, and NPAHD.

semicarbazide (SEM, a metabolite of nitrofurazone) because it is believed through research that parent drugs metabolize rapidly in vitro and in vivo, whereas the protein-bound metabolites of these nitrofurans are detectable for a long time after administration. It is, however, almost impossible to regulate the animal food production industry in order to detect residues of nitrofurans effectively in thousands of tons of food animal products. One option to resolve the problem is to enforce regulation from the very early stage. The illegal users of nitrofurans in animal production often add drugs in the feed and drinking water for animals. Therefore, there is a need to detect parent drugs for nitrofurans in animal production. In this study, we prepared the antibody against nitrofurantoin by using an immunogen prepared from the conjugation of 3-carboxybenzaldehyde (CBA), AHD, and bovine serum albumin (BSA). The antibody obtained shows excellent specificity and sensitivity toward the parent drug nitrofurantoin due to the structural similarity between AHD and nitrofurantoin. On the basis of these results, we developed an immunoassay to detect nitrofurantoin residues in water for the first time.

MATERIALS AND METHODS

Chemicals and Materials. Nitrofurantoin, furaltadone, furazolidone, nitrofurazone, 1-aminohydantoin hydrochloride (AHD), semicarbazide (SEM), 5-methylamorpholino-3-amino-2-oxazolidinone (AMOZ), *o*-nitrobenzaldehyde (*o*-NBA), bovine serum albumin (BSA), ovalbumin (OVA), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), and Freund's complete (cFA) and incomplete adjuvants (iFA) were purchased from Sigma-Aldrich (St. Louis, MO). *o*-Phenylenediamine (OPD) was purchased from Xinjingke Biotechnology (Beijing, China). Goat anti-rabbit IgG−horseradish peroxidase conjugate was provided by Military Medical Institute (Beijing, China). Tri-*n*-butylamine and glutaric dialdehyde were obtained from Chinese Medicine Shanghai Holding Co. Ltd. Isobutyl chloroformate was provided by Feixiang Chemical Co. (Shanghai, China). 3-Carboxybenzaldehyde (CBA) was purchased from Dianyao Chemical Co. (Shanghai, China). 3-Amino-2-oxazolidinone (AOZ) was a generous gift from Dr. Jianwu Wang's laboratory (Shandong University, Jinan, China). Dimethylformamide (DMF), methanol, ethylenediamine dihydrochloride (EDA), hydrogen peroxide (30%), and other reagents used were of chemical grade from Guangmang Chemical Co. (Jinan, China).

Instrumentation and Supplies. ELISA was performed in polystyrene 96-well microtiter plates (Bio Basic Inc.) and spectrophotometrically read with an automatic microplate reader KHB ST-360 from Shanghai Zhihua Medical Instrument Ltd. UV data were collected on a U-4100 spectrophotometer from Hitachi Co. Centrifugation was carried out with a refrigerated centrifuge (Biofuge stratos, Heraeus). ^{1}H NMR was measured in a 300 MHz Bruker instrument. Protein dialyses were performed using dialysis tubes from Aibo Economic and Trade Co., Ltd. (Jinan, China). Rotary evaporation was carried out with the rotary evaporator (RE-5203A) from Shanghai Zhenjie Laboratory Instrument Co. Ltd.

Buffers. For the preparation of all buffers and reagents for the immunoassays, ultrapure deionized water was used. Phosphate-buffered saline (PBS, pH 7.4) consisted of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl. The wash buffer (PBST) was a PBS buffer containing 0.05% Tween 20. As a coating buffer, 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) was used. The blocking buffer was PBS + 1% of OVA + 0.05% (v/v) Tween20. The substrate buffer was 0.1 M sodium acetate/citrate buffer (pH 5.0). To prepare the substrate solution, 10 mg of OPD solution was dissolved in 25 mL of sodium citrate buffer and this solution plus 5 μL of H_2O_2 (30%). The stopping solution was 2 N HCl.

Preparation of cBSA and cOVA. In this procedure, carboxylic acid groups of the carrier proteins of BSA and OVA were converted into primary amine groups with an excess of EDA. A solution of 1 g of BSA (0.015 mmol) and 56 mg of EDC (0.300 mmol) in 20 mL of PBS (0.1 M, pH 7.4) was added slowly into a solution of 18 mg of EDA (0.300 mmol) in 20 mL of PBS (0.1 M, pH 7.4) with continuous stirring. The mixture solution was incubated continuously for 2 h at room temperature and then dialyzed [molecular weight cutoff (mwco) of 12000−14000] under stirring against PBS (0.1 M, pH 7.4) to remove free EDA. Cationized BSA and OVA were defined as cBSA and cOVA, respectively. The solution was lyophilized, and the white solid (cBSA) obtained was stored at −20 °C before used in the next reaction (0.972 g, 97.2%). The cationized OVA (cOVA) was prepared in a similar method (0.886 g, 88.6%).

Syntheses of CPAHD and NPAHD. In this procedure, 75 mg (0.5 mmol) of CBA was dissolved in 10 mL of methanol followed by the addition of 76 mg (0.5 mmol) of AHD·HCl. The reaction mixture was refluxed overnight at 65 °C under stirring. Thin-layer chromatography (TLC) was used to monitor reaction progress. After the reaction was over according to TLC, the mixture was washed with ethanol three times to remove unreacted CBA (86 mg, 70%). ^{1}H NMR demonstrated that CBA was derivatized successfully. The conjugate of CBA and AHD obtained was named CPAHD. NPAHD [abbreviated name for the conjugate of *o*-nitrobenzaldehyde (NBA) and AHD] was prepared in a similar manner (93 mg, 75%). CPAHD: ^{1}H NMR (DMSO, 20 °C, δ vs TMS) 4.367 (s, 2H, CH2), 7.536−7.646 (m, 1H, C6H), 7.896−7.999 (m, 2H, C6H2), 8.308−8.329 (m, 1H, C6H), 7.936 (s, 1H, CH═), 11.308 (s, 1H, NH), 13.163 (s, 1H, COOH). NPAHD: ^{1}H NMR (DMSO, 20 °C, δ vs TMS) 4.359 (s, 2H, CH2), 7.646−7.697 (m, 1H, C6H), 7.792−7.842 (m, H, C6H), 8.007−8.082 (m, 2H, C6H2), 8.031 (s, 1H, CH═), 11.383 (s, 1H, NH).

Preparation of Immunogen (CP-AHD-cBSA). The immunogen of CP-AHD-cBSA was prepared via a mixed acid anhydride reaction similar to that reported by Cooper et al. (*8*). In this procedure, 50 μL (0.2 mmol) of tri-*n*-butylamine was added to a solution of 40 mg (0.14 mmol) of CPAHD in 10 mL of dry DMF. The mixture was stirred for 10 min at room temperature, followed by the dropwise addition of 87 μL (0.67 mmol) of isobutyl chloroformate in the ice bath. The reaction mixture was allowed to stand for 1 h at room temperature. Subsequently, activated CPAHD was added very slowly under stirring to a solution of 80 mg (1.2 μmol) of cBSA dissolved in 10 mL of PBS and then

stirred for 4 h at room temperature. The reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.01 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten. The precipitate was removed by centrifugation at 3000*g*, and the supernatant was lyophilized to obtain the conjugate of CP-AHD-cBSA, which was stored at −20 °C for future use (64 mg, 80%).

Preparation of Coating Antigens (AHD−Glutaric Dialdehyde−cOVA). In this procedure, 117 mg (1.2 μmol) of cOVA was dissolved in 15 mL of sodium borate (0.5 M, pH 8.5). Subsequently, 16 mg (0.1 mmol) of AHD·HCl was added to the solution, followed by the dropwise addition of 0.2 mL of glutaric dialdehyde (25%) at 4 °C with constant stirring. The color of the mixture solution slowly changed to yellow. The reaction was incubated for 2 h at 4 °C. The yellow reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.01 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten. The solution was lyophilized to obtain a yellow conjugate of AHD−glutaric dialdehyde−cOVA, which was stored at −20 °C for future use (97 mg, 83%).

Immunization of Rabbits. Two male New Zealand white rabbits were subcutaneously immunized at multiple sites on the back with CP-AHD-cBSA conjugate. The initial immunization was a subcutaneous injection with 1 mg of conjugate in 0.5 mL of NaCl (0.9%) and 0.5 mL of cFA. Subsequent booster injections [0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of iFA] were performed 15 days later and then at 12 day intervals. One week after each booster, serum titers were determined in ELISA. The antiserum obtained after each booster was prepared by allowing the blood to clot overnight at 4 °C followed by centrifugation to remove blood cells and particulate material. Ten days after the last booster, all rabbits were then exsanguinated by heart puncture under general anesthesia and euthanized by lethal injection. The serum was separated from blood cells by storage of the blood overnight at 4 °C and centrifuged at 13000 rpm/min for 20 min. The crude serum obtained was purified by saturated ammonium sulfate (SAS) precipitation method [purified three times using 50, 33, and 33% (v/v) of SAS, respectively], and sodium azide was added as a preservative at a final concentration of 0.02% (w/w). The purified serum was then aliquotted and stored at −70 °C for future use.

Antibody Titer Determination by Indirect ELISA. The titer of the antibody was tested by indirect ELISA, using a procedure described below. The microplates were coated with coating antigen AHD−glutaric dialdehyde−cOVA at 5 μg/mL (50 μL/well) by overnight incubation at 4 °C. Plates were washed with washing buffer three times and blocked with 250 μL/well of blocking buffer containing OVA, followed by incubation for 1 h at room temperature. Plates were washed for three times again, the appropriate dilution of the antisera was added, and the plates were incubated for 2 h at room temperature. Plates were washed three times, and goat anti-rabbit IgG−HRP (1:3,000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. Plates were washed three times, OPD substrate solution was added (50 μL/well), and the plates were incubated for another 30 min at room temperature. The color was developed by adding HCl stopping solution (50 μL/well), and absorbances were measured at 492 nm. Absorbances were corrected by blank reading. The serum collected prior to immunization was used as negative control, and the antibody titer was defined as the reciprocal of the dilution that results in an absorbance value that is twice the background.

Development of Indirect Competitive ELISA. The checkerboard procedure was used to optimize the coating antigen and the primary antibody concentrations. To each well of a 96 well plate was added 50 μL of 5 μg/mL of AHD−glutaric dialdehyde−OVA solution in bicarbonate buffer (0.05 M, pH 9.6), and incubation was performed overnight at 4 °C. The plate was washed with washing buffer three times and blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. After removal of the blocking solution and three washings of the plate, 100 ng of primary antibody was added to each well followed by buffer or the analytes in buffer, and the plate was incubated for 2 h. The plate was washed three times, and goat anti-rabbit IgG−HRP (1:3000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. The plate was washed three times, OPD substrate solution was added (50 μL/well), and the plate was incubated for another 30 min at room temperature for color development. The HCl stopping solution (50 μL/well) was added, and absorbances were measured at 492 nm. Absorbances were corrected by blank reading. Preimmune withdrawn serum was used as negative control. The percent inhibition was calculated as

$$\% \text{ inhibition} = [1 - (OD_{492,B}/OD_{492,NC})] \times 100$$

where $OD_{492,B}$ is the absorbance with various concentrations and $OD_{492,NC}$ is the absorbance for the negative control.

Specificity Determination. Competitive immunoassays were performed using various compounds structurally related to nitrofurantoin, to determine the respective IC_{50} value and cross-reactivity. ELISA plates were coated with coating antigen at 5 μg/mL (100 μL/well) by incubation overnight at 4 °C and then washed three times. The plates were blocked (250 μL/well) for 2 h at room temperature and washed three times again. For the competition step, 50 μL/well of competitors was added at a concentration ranging from 0.01 to 1000 ng/mL and co-incubated with 50 μL/well of primary antibody for 2 h. The secondary antibody and color development were the same as described above. After reading the plate, the IC_{50} value was determined by using the concentration of inhibitor that leads to a 50% decrease of the maximum signal; the cross-reactivity (percent) was calculated as $(IC_{50,\text{nitrofurantoin}})/(IC_{50,\text{compound}}) \times 100$.

Standard Curve Generation. The AHD−glutaric dialdehyde−cOVA (5 μg/mL) was used as a coating antigen, and indirect competitive ELISA was performed as described above. The selected antisera at 1:5000 dilution were utilized as primary antibody and co-incubated with nitrofurantoin. The standard calibration curve with final nitrofurantoin concentrations of 0.5, 1.0, 2.0, 5.0, 10, and 20 ng/mL was run in PBST.

Inter- and Intra-assay Variation Determination. The water was fortified by nitrofurantoin at final concentrations of 0.5, 1.0, 2.0, 5.0, 10, and 20 ng/mL. Interassay variation was computed from the analysis of five replicates of each dilution carried out on five different days. Intra-assay variation was measured by analysis of five replicates of each dilution on a single day. Sample recoveries were determined from a standard curve.

RESULTS AND DISCUSSION

Immunogen Design. To develop an antibody that is specific to nitrofurantoin, the design of the corresponding immunogen is very important. From the structure of nitrofurantoin (**Figure 1**), it can be seen that the furan moiety is shared by all four nitrofurans listed including furazolidone, furaltadone, nitrofurantoin, and nitrofurazone; thus, the furan moiety cannot be used as specific structural moiety when in the design of an immunogen for nitrofurantoin. The AHD moiety, however, is a specific part for nitrofurantoin and is suitable for use as part of an immungen to develop a specific antibody toward nitrofurantoin. The preferred approach would be to apply the whole molecule of nitrofurantoin as the starting chemical to connect it with carrier proteins. It is, however, hard to find a suitable point in the furan moiety in nitrofurantoin to link with carrier proteins. At first, we considered the possibility of reducing the NO_2 group to NH_2 group by using a reducing agent such as H_2 or Fe/HCl systems, but it is a concern that the reduction process might damage the whole molecule of nitrofurantoin. Currently, we are still seeking a way to apply a reduction procedure to prepare an immunogen using the whole molecule of nitrofurantoin. On the basis of this consideration, in this research, we focus on synthesizing an immunogen using AHD as a starting chemical. Inspired by previous research from Kennedy's group (*5*−*8*), we modified AHD by a similar strategy used for AOZ by using 3-carboxybenzaldehyde to activate AHD to bring in a carboxylic acid group that can be conveniently linked to carrier proteins. The conjugate formed is named CPAHD (**Figure 1**).

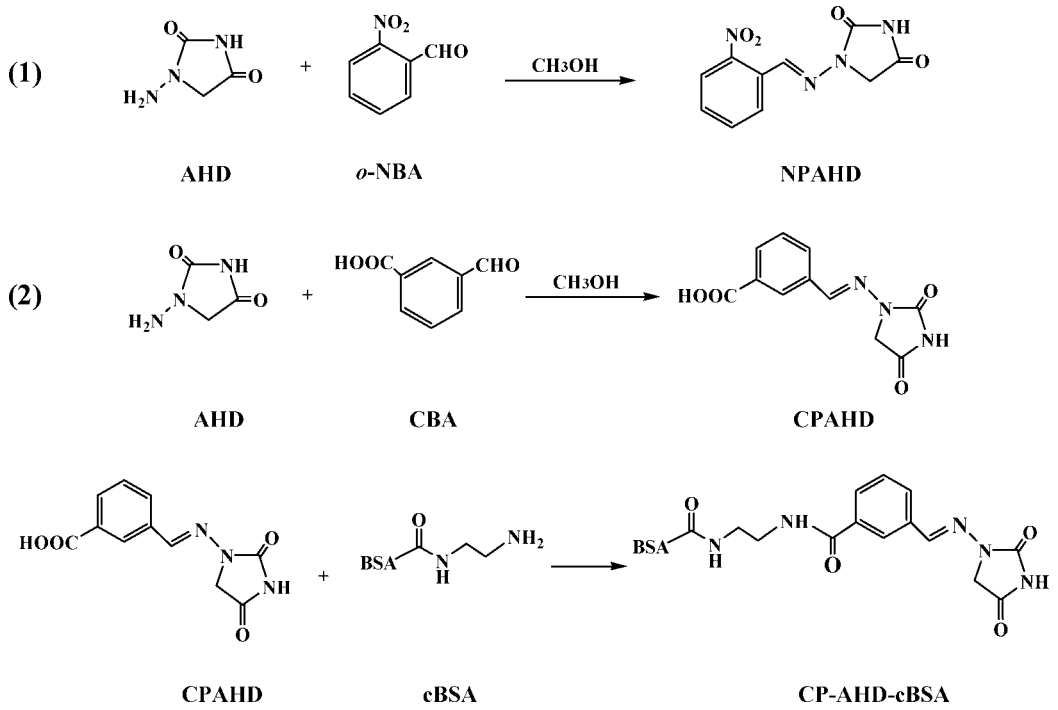

Figure 2. Synthetic procedure to prepare NPAHD (1) and immunogen CP-AHD-cBSA (2).

NPAHD also was prepared in this study because it is an important derivative used in HPLC and LC-MS/MS to detect residues of AHD. We want to know the cross-reactivity of NPAHD with the antibody being prepared. ^{1}H NMR spectroscopy clearly demonstrates the successful formation of CPAHD and NPAHD (see Materials and Methods for detailed information of ^{1}H NMR results). Among the carrier proteins, BSA and OVA are the two most often used carrier proteins, and usually they gave satisfactory results. To convert carboxylic acid groups on the carrier protein to primary amine groups, BSA and OVA were treated with an excess of EDA as described previously (*11*, *12*). The cationized BSA (cBSA) prepared has a higher linking capacity with CPAHD due to more primary amine groups being available on cBSA than on BSA (*12*). Moreover, the use of cationized carrier proteins can minimize cross-linking and increase their p*I* to generate more immune response compared to their native forms (*13*). Quite a few methods can be used to prepare immunogens when one has a hapten with a carboxylic acid group in it. The optimal result was achieved by applying the mixed acid anhydride method to link a free carboxylic acid group in CPAHD with amino groups in cBSA. This reaction results in an amide bond between CPAHD and the carrier protein (**Figure 2**).

Antibody Characterization. The titer of antibody was determined by indirect ELISA as >512000 for both rabbits used in the immunization process with the titer being defined as the reciprocal of the dilution that results in an absorbance value that is twice the background. A representative inhibition curve for nitrofurantoin is shown in **Figure 3**. The IC_{50} of nitrofurantoin is at the parts per billion level (3.2 ppb), indicating excellent sensitivity of the antibody. There is no general agreement for the calculation of assay sensitivity for a competitive immunoassay. Fleeker reported a limit of detection (LOD) of an assay to be 3 times the standard deviation of the negative control from its mean absorbance (*14*, *15*), whereas Midgley et al. calculated the LOD as the concentration that corresponds to 10% inhibition in inhibition curve (namely, 90% of B/B_0) (*16*). There are also some researchers using 10 times the standard deviation of the blank as the LOD. We calculated the LOD using these three

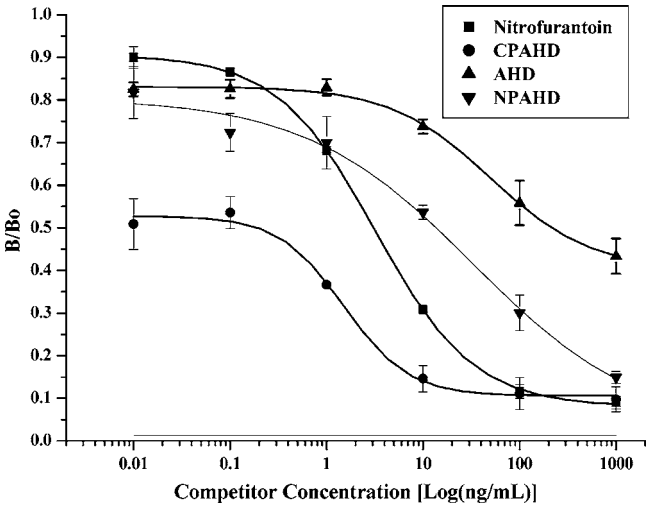

Figure 3. ELISA curves for polyclonal antibody using nitrofurantoin (■), CPAHD (●), AHD (▲), and NPAHD (▼) as competitors in PBS buffer solution. Each point represents the average of five replicates. Nitrofurantoin shows an excellent ELISA curve with an IC_{50} of 3.2 ppb.

methods and obtained three LOD numbers as 0.18 (Fleeker method), 0.1 (Midgley method), and 0.2 ppb (10 × SD of the blank method). To be conservative, 0.2 ppb was chosen as the LOD for the assay developed in this study.

Specificity of the Antibody. The specificity of the developed assay was evaluated by determination of the cross-reactivity toward selected compounds related to this research. The cross-reactivity studies were carried out by an indirect competitive ELISA by adding various free competitors at different concentrations (from 0.01 to 1000 ppb) to compete with binding of the antibody to the coating antigen. Cross-reactivity was measured by comparison of the IC_{50} produced by the competitor with nitrofurantoin. The antibody shows excellent specificity with significant cross-reactivity only toward CPAHD and NPAHD (**Table 1**). It is understandable that the antibody shows a high cross-reactivity toward CPAHD because this is a modified hapten used to link to carrier protein cBSA to prepare the

Table 1. IC_{50} Values and Cross-Reactivities of Selected Compounds

compound	IC_{50}[a] (ng/mL)	cross-reactivity[b] (%)
nitrofurantoin	3.2	100
CPAHD	0.2	1600
NPAHD	15	21
AHD	260	1.2
nitrofurazone	>5000	<0.1
furazolidone	>5000	<0.1
furaltadone	>5000	<0.1
AMOZ	>5000	<0.1
CBA	>5000	<0.1
NBA	>5000	<0.1
NPSEM	>5000	<0.1
NPAOZ	>5000	<0.1
AOZ	no competition	
SEM	no competition	

[a] IC_{50} was the competitor concentration at which the absorbance value was decreased by half compared to the absorbance value of no competitor. Data represent three separate experiments run on three different days. For this antibody, only nitrofurantoin, CPAHD, NPAHD, and AHD show significant cross-reactivity. [b] Percentage of cross-reactivity is defined as the ratio of the nitrofurantoin concentration (ng/mL) at IC_{50} to that of the test compound × 100.

Table 2. Inter- and Intra-assay Variations of Water Spiked with Nitrofurantoin

	interassay[a]				intra-assay[b]			
level (ppb)	*n*	measured (ppb)	recovery (%)	CV (%)	*n*	measured (ppb)	recovery (%)	CV (%)
0.5	5	0.49 ± 0.03	98	6.1	5	0.48 ± 0.03	96	6.2
1	5	0.88 ± 0.1	88	11.4	5	0.96 ± 0.06	96	6.2
2	5	1.76 ± 0.08	88	4.5	5	1.81 ± 0.06	91	3.3
5	5	5.17 ± 0.36	103	7.0	5	5.17 ± 0.14	103	2.7
10	5	9.57 ± 0.82	96	8.6	5	9.0 ± 0.24	90	2.7
20	5	18.22 ± 0.56	91	3.1	5	18.2 ± 0.87	91	4.8

[a] Interassay variation was determined by five replicates on 15 different days. [b] Intra-assay variation was determined by five replicates on a single day.

immunogen. It is well-known that antibodies elicited to haptenic conjugates show a preferential recognition to the part of the molecule that is farthest from the attachment site of the hapten to the carrier protein (*17*−*19*). This is certainly true considering previously published results related to antibody design and cross-reactivity studies (*20*, *21*). In this research, however, the moiety close to the linking point also plays an important role in the specificity of the antibody if the structures of CPAHD and NPAHD are compared. Both CPAHD and NPAHD have moieties composed of the condensation of AHD and benzaldehyde, but their cross-reactivities are very different (1600% for CPAHD and 21% for NPAHD). Considering that their only structural differences are the substituents in the benzene ring, we believe that the phenyl moiety plays a certain role for antibody recognition despite this part being close to the linking point of hapten and carrier protein.

Detection of Nitrofurantoin Residue in Water. Nitrofurantoin is often used illegally by adding it to drinking water fed to animals. Therefore, we used water as a real system to measure residues of the drug. The results are shown in **Table 2**. The recovery rates are in the ranges of 88−103% for interassay and 90−103% for intra-assay. The CVs are in the ranges of 3.1−11.4% for interassay and 2.7−6.2% for intra-assay. Compared with other biological matrices such as liver, meat, and milk, the water system is relatively simple without influence from biological substances. The results obtained are shown in **Table 2**.

In summary, we designed and prepared an antibody to a derivative of 3-aminohydantoin for the first time. The antibody showed excellent specificity toward nitrofurantoin, the parent compound for AHD, with IC_{50} in the parts per billion range of 3.2 ppb and a limit of detection of 0.2 ppb. Using the water system, the immunoassay developed has shown satisfactory results. We believe that this ELISA assay can be used as a screening method to detect residues of nitrofurantoin in the drinking water for animals to enforce regulation.

ABBREVIATIONS USED

MRL, maximum residue level; BSA, bovine serum albumin; EDA, ethylenediamine dihydrochloride; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; *o*-NBA, *o*-nitrobenzaldehyde; CBA, 3-carboxybenzaldehyde; CPAHD, conjugate of CBA and AHD; NPAHD, conjugate of NBA and AHD; IC_{50}, concentration at 50% inhibition; IC_{10}, concentration at 10% inhibition; PBS, phosphate-buffered saline; DMF, *N*,*N*-dimethylformamide; cFA, complete Freund's adjuvant; iFA, incomplete Freund's adjuvant.

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