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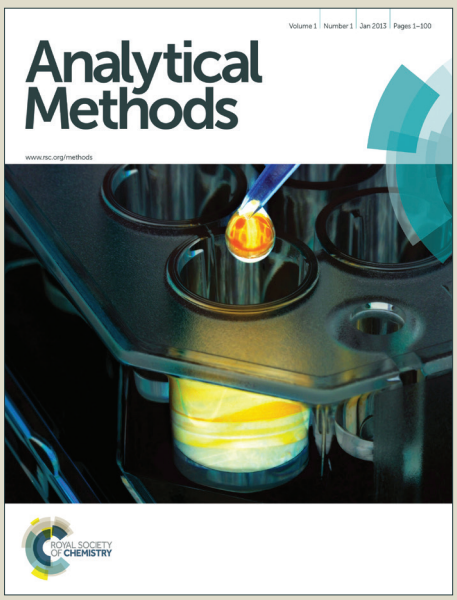

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Development of a direct competitive chemiluminescent ELISA for the detection of nitrofurantoin metabolite 1-amino-hydantoin in fish and honey

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Abstract: A direct competitive enzyme-linked immunosorbent assay (ELISA) with chemiluminescent (dcCLELISA) detection for the 1-amino-hydantoin (AHD) was developed in this study. AHD was derivatised with 4-carboxybenzaldehyde to produce 1-[(4-Carbo-benzylidene)-amino]-imida-zolidin-2,4-dione(CPAHD). Monoclonal antibodies (MAb) against AHD were prepared through immunization of BALB/c mice with synthesized CPAHD-Jeffamine-BSA as an antigen. Luminol, *p*-iodophenol, and urea peroxide mixture solution served as the substrate in CLELISA. The specificity of the MAb, estimated as the cross-reactivity values from the dcCLEILISA assay for 1-[(4-nitro-benzylidene)-amino]-imidazolidin-2,4-dione (NPAHD) and CPAHD was 100% and 39.67%, respectively. Other compounds all showed less than 0.01%. The sensitivity of the antibody, estimated as the IC_{50} value, was 0.60 μg L^{-1}. The limits of detection for dcCLELISA in fish and honey samples were 0.1 and 0.28 μg kg^{-1}, respectively, and the mean recovery values ranged from 83.6% to 94.7% for fortified samples at levels of 0.25-10 μg kg^{-1} with coefficient of variation values below 15%. Finally, dcCLELISA was compared to a commercial kit in the detection of AHD in spiked fish and honey samples. The immunoassay method described here showed a broad detection range and high sensitivity. It could be used for high-throughput monitoring of AHD in fish and honey samples and possibly other types of food.

Keywords: Chemiluminescent ELISA; monoclonal antibody; AHD; residual detection

1. Introduction

Nitrofurantoin belongs to the nitrofuran group of antibiotics, all of which have a characteristic 5-nitrofuran ring. Nitrofurans (nitrofurantoin, furazolidone, furaltadone, and nitrofurazone) have been widely and effectively used in the treatment of gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. in cattle, poultry, and pigs. They have also been frequently used as growth promoters in animal husbandry [1, 2].

Nitrofurantoin, furazolidone, furaltadone, and nitrofurazone are rapidly metabolized to 1-amino-hydantoin (AHD), 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), and 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ), respectively, within a few hours of administration. These metabolites bind to tissue proteins and persist after treatment in animal tissues for considerable periods. In this way, it is feasible to monitor residues of nitrofurans by detecting their tissue-bound metabolites which are released by mild acid hydrolysis[3]. Usually, these metabolites are derivatised with o-nitrobenzaldehyde (o-NBA) to form

nitrophenyl (NP) derivatives to increase its molecular mass prior to the detection[4]. In this study, NPAHD was used as a inhibitor in screening of positive hybridoma cells during preparation of monoclonal antibody.

Many studies have reported that nitrofurans and their metabolites may have carcinogenic and mutagenic effects[5-7]. For this reason, nitrofurans have been banned from use in food-producing animals in the European Union (E.U.) since 1995 [8]. The EU Commission Decision of 13 March 2003 had set a minimum required performance limits (MRPLs) at 1 μg kg^{-1} (for each nitrofuran metabolite) with any methods dealing with the assessment of nitrofuran levels in poultry meat and aquaculture products[9]. Strict prohibitions on the use of nitrofurans in animal husbandry were also put in place in the USA and China [10,11].

Analytical methods for the detection of AHD in animal tissues have mainly been based on LC/UV, LC-MS, and LC-MS/MS[12-15] with the LOD between 0.1-0.29 μg kg^{-1}. However, these methods require expensive equipment and considerable amounts of time. In contrast, the enzyme-linked immunosorbent assay (ELISA) provides an alternative inexpensive, sensitive, and fast screening method of detecting AHD. Xu Developed an immunochromatographic assay for rapid detection of AHD in urine specimens with the detection limit of 10 μg L^{-1} and high specificity[16]. Liu detected AHD in water using the polyclonal antibodies of AHD by an indirect competitive ELISA with the IC_{50} of 3.2 μg L^{-1} and the detection limit of 0.2 μg L^{-1}[17]. Chemiluminescent ELISA (CLELISA) may improve the sensitivity of immunoassays by at least 2-3 orders of magnitude relative to conventional colorimetric detection[18]. To the best of our knowledge, there are no reported CLELISA methods to detect AHD in animal tissues. In most immunoassays, nitrofuran metabolites were derivatised with 3-carboxybenzaldehyle (3-CBA) or 4-carboxybenzaldehyle (4-CBA) to form immunizing haptens[10,19-25]. The haptens were coupled to carrier proteins through the carboxylic acid spacer to generate immunogens. However, in relation to hapten design of small-molecular-weight compounds, a suitable length of spacer between the hapten and the carrier protein can facilitate production of the desired antibodies[26,27]. Inspired by these previous reports, AHD was derivatised with 4-carboxybenzaldehyde to produce CPAHD and the immunogen was prepared using Jeffamine as a spacer between CPAHD and the carrier protein. The ability of the novel immunogen to produce specific antibodies against AHD was

evaluated and compared to the traditional immunogen, which is derivatised from AHD without Jeffamine. In the present study, a direct competitive enzyme-linked immunosorbent assay (ELISA) combined with chemiluminescent (dcCLELISA) was developed for screening AHD residues in fish and honey. The method developed in the present study was compared to a commercial kit designed to detect AHD in spiked samples. The results indicated that the sensitivity of the new method was higher than that of the commercial kit.

2. Materials and methods

2.1Materials and equipment

1-amino-hydantoin (AHD) was purchased from DaRui Shanghai Industrial Co. Ltd. (Shanghai, China). Ovalbumin (OVA) and 4-nitrobenzaldehyde (4-NBA) were obtained from Heng Ye Zhong Yuan Chemical Industrial Co., Ltd (Beijing, China), 4-carboxybenzaldehyde (4-CBA) was obtained from Accela ChemBio Co., Ltd (Shanghai, China), complete Freund's adjuvant, incomplete Freund's adjuvant, bovine serum albumin (BSA) and polyethylene glycol-6000 (PEG) were purchased from Sigma company (USA). N, N-dimethylformamide (DMF), N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tetramethyl benzidine (TMB) and goat-anti-mouse IgG labeled with horseradish peroxidase (HRP) were purchased from Zhenyu Bio-Technology Co., Ltd. (Shanghai, China). Commercial kit (RIDASCREEN® Nitrofuran AHD) was purchased from R-biopharm AG (Germany). The bag filter (DM20/DM25) was purchased from Siji Bio-Products Co., Ltd. (Shanghai, China). Chemiluminescence was measured with a BioTek Synergy Microplate Luminometer (BioTek, USA). The microplate reader (ELX800) was purchased from BioTek (USA), and the 96-well polystyrene microtiter plates were purchased from Costar (USA).

2.2 Buffers and solutions

The coating buffer was 0.05 M carbonate solution (pH 9.6). Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was used for antibody dilution. The blocking buffer was 1% gelatin in PBS (w/v). The washing solution was PBS buffer containing 0.05% tween 20 (v/v). PBS with 5% fetal bovine serum was used for peroxidase-conjugated affinipure goat anti-mouse

IgG dilution. Chemiluminescence substrate solution was sodium borate buffer (0.05 M pH 9.0) containing luminol (2mmol), *p*-iodophenol (0.25mmol), and urea peroxide (4mmol).

2.3 Preparation of AHD derivative

CPAHD was derivatised from AHD with 4-CBA via a condensation reaction as shown in Figure 1. 5 ml of DMF was added to 0.65 g of 4-carboxybenzaldehyde in 6 ml of water with stirring and then 0.45 g of AHD was added. The mixture was stirred at room temperature for 2 h and then filtered. The pale yellow solid layer was washed with water for three times and dried. The synthesized compound was confirmed by ^{1}HNMR spectroscopy. NPAHD was prepared in a similar manner as for CPAHD.

2.4 Preparation of CPAHD conjugates

Firstly, 20 mg of BSA or OVA was dissolved in 2 ml of 0.05 M MES solution. Then DCC (5 mg) and NHS (3 mg) were added. After the compounds had dissolved sufficiently, 15 µl of Jeffamine was added and stirred gently at room temperature for 4 h and the conjugates were dialyzed against 1×PBS (pH 7.4) for 3 d. Then CPAHD was coupled to Jeffamine-BSA and Jeffamine-OVA for immunogen and coating antigen, respectively, according to the method described by Jiang [25]. The details of the method of synthesis are as follows. Firstly, CPAHD (14 mg), NHS (10 mg) and DCC (15 mg) were dissolved in 2 ml of DMF and the mixture was stirred gently overnight at room temperature. After the mixture was centrifuged at 2500 g for 10 min, the supernatant was added dropwise to Jeffamine-BSA or Jeffamine-OVA in 2 ml of 0.05 M PBS (pH 7.4) and stirred 12 h at 4°C. The eluted conjugates were dialyzed against PBS (pH 7.4), freeze-dried, and stored at 4°C. Full wavelength (200-500 nm) UV-vis scanning was used to confirm the structures of the final conjugates.

CPAHD-HRP conjugation was performed using a modified version of a previously described method[28]. Briefly, CPAHD (2.5 mg), DCC (4 mg), and NHS (1.8 mg) were dissolved in 0.25 ml of DMF and stirred gently for 1 h at room temperature. Then the mixture was added dropwise to 2.5 mg of HRP in 1.5 ml of 0.1 M pH 8.0 PBS. After that 1.91 mg of EDC was added to the mixture and stirred gently for 2 h at room temperature and the reaction mixture was dialyzed against PBS (pH 7.4) for 3 d. The prepared conjugates were diluted with glycerol and stored at -20 °C until needed.

2.5 Immunization and production of the monoclonal antibody

Four 10-week old BALB/c mice were immunized with 120 μg of CPAHD-Jeffamine-BSA in complete Freund's adjuvant. Every two weeks, the mice were given booster immunizations at the same dosage of immunogen emulsified in Freund's incomplete adjuvant. Blood samples were taken from tail veins at 10 days after the fourth immunization and tested for antibody production by direct ELISA. The mouse exhibiting the highest titer was subjected to final immunizations with CPAHD-Jeffamine-BSA without adjuvant through injection into the peritoneal cavity.

Three days later, the mouse with the highest titer was sacrificed and the spleen was collected for cell fusion. Spleen cells from the mouse and myeloma cells were fused at a ratio of 1:10 in 1 ml of 50% PEG. After cell fusion, the cells were cultured using selection medium containing 20% fetal calf serum and hypoxanthine (0.1mmol), aminopterin (0.04mmol) thymidine (0.16mmol) (HAT) in microtiter polystyrene plates. After 7 days, the growing hybridoma cells were screened for antibody production using an indirect ELISA method. Then the positive hybridoma cells were further selected by indirect competitive ELISA according to the reported method[23]. And the indirect competitive ELISA was used to screen for antibodies specific for NPAHD and to evaluate the sensitivity. The positive hybridomas were subcloned by the limiting dilution method. Stable antibody-producing clones were expanded until monoclones were obtained. Then, monoclonal antibodies were produced in mouse ascites and purified with saturated ammonium precipitation.

2.6 dcCLELISA and icCLELISA optimization

In the current study, two CLELISA assays (i.e., dcCLELISA and icCLELISA) were independently developed, evaluated and optimized for the detection of AHD residues.

In dcCLELISA the concentrations of coating antibody and CPAHD-HRP were optimized using the checkerboard method [29]. And the high binding white opaque plates were coated with antibody at dilutions of 1:125, 1:250, 1:500, 1:1000, and 1:2000 and the rows of these plates were given different dilutions of CPAHD-HRP. The dcCLELISA was performed as follows: Firstly, high-binding white opaque plates (Shanghai GenoMintel Medical Instrument Co., Ltd.) were coated with 100 μl of monoclonal antibody of CPAHD overnight at 4°C. Then plates were washed with PBST and blocked with 200 μl blocking buffer to each

well for 2 h at 37°C. Then 50 µl of the standard or sample solution and 50 µl of CPAHD-HRP were added to each well and the plates were incubated at 37 °C for 30 min. They were then washed again, and 100 µl of prepared chemiluminescence substrate mixture was added to each well. Chemiluminescence was measured with a fluorescence microplate reader within 40 min of the addition of the substrate.

A calibration curve was constructed in the form $(B/B_0) \times 100\%$ vs. logC, where B and B_0 is the relative light unit (RLU) of the analyte at the standard point and at zero concentration of the analyte, respectively. The concentrations of NPAHD in the samples were calculated using the standard curve run in the same microtiter plate.

In icCLELISA the concentrations of coating antigen and antibody were also optimized using the checkerboard method[29]. And the high-binding white opaque plates were coated with coating antigen (CPAHD-Jeffamine-OVA) at concentrations of 4, 2, 1, 0.5, 0.2, 0.1 µg ml^{-1} and rows of these plates were given different dilutions of antibody. icCLELISA procedures were then performed as follows: Firstly, 96-well high-binding white opaque plates were coated overnight with coating antigen solution. Then plates were washed with PBST and blocked for 2 h with 200 µl blocking buffer in each well at 37°C. Then 50 µl of the 4-NPAHD standard solution and appropriate concentrations of monoclonal antibody diluted in PBST buffer solution containing 10% fetal bovine serum were added to each well and the plate was incubated at 37°C for 30 min. Then the plates were washed three times and 100 µl of 4000-fold diluted goat anti-mouse IgG-HRP was added to each well and incubated for 1 h at 37°C. The subsequent procedure was similar to those described in the dcCLELISA.

2.7 Sample preparation

The samples were prepared using a modified version of the method previously described by Pimpitak [23]. Firstly, fish (common carp) homogenate and honey (1.00±0.01 g) were weighed into disposable plastic centrifuge tubes and fortified with 50 µl of AHD at different concentrations in 4 ml of deionized water. Then 0.5 ml of 1 M HCl and 100 µl of 10 mM 4-nitrobenzaldehyde in DMSO were added to the homogenized tissue solutions. Each sample was thoroughly mixed and incubated for 3 h in a water bath at 55 °C. The mixture was allowed to room temperature and 5 ml of 0.1 M dibasic potassium phosphate was added.

Then 0.4 ml of 1 M NaOH was added to the mixtures to adjust the pH to 7.4. After that, 6 ml of ethyl acetate was added to the samples, which were vortexed vigorously for 1 min and centrifuged at 4000 g for 10 min. A volume of 3 ml ethyl acetate supernatant was removed, placed in centrifuge tubes, and evaporated until dry in a heating block at 50°C in a nitrogen environment. Residues were dissolved in 2 ml of a 1:1 (v/v) mixture of n-hexane and 0.1 mol L^{-1} PBS (pH 7.4) and vortexed thoroughly for 1 min. After centrifugation at 4000 g for 5 min, the buffer phase was separated and collected for detection.

2.8 Assay performance

The limit of detection (LOD) was determined based on the mean value of 20 blank samples plus three-times-the-mean standard deviation. The accuracy and precision of the method were represented by recovery and coefficient of variation (CV), respectively. Recovery % = concentration measured/concentration fortified ×100%. The blank samples were fortified with 0, 0.25, 0.5, 2.5, and 10 μg kg^{-1} of AHD, and the recovery was calculated after determination. In order to assess CV, the assay was repeated three times. The mean recovery and CV values were calculated for two kinds of food samples. The precision of the CLELISA method was analyzed by repeated analysis of the fortified samples and comparison of the intra- and inter-assay CVs. Intra-assay CV was measured by three replicates of each fortified concentration. And the inter-assay CV was based on the results of five different days.

The 50% inhibitory concentration (IC_{50}), represents the concentration of an inhibitor that is required for 50% inhibition binding of the antibody to the coating antigen. Competition curves were graphed by plotting the maximum antibody binding (%) against the logarithm of the analyte concentration.

Antibody specificities were determined through cross-reactivity experiments. AHD, CPAHD, NPAHD, AOZ, CPAOZ, NPAOZ, AMOZ, CPAMOZ, NPAMOZ, SEM, CPSEM, NPSEM, CP, NP, nitrofurantoin, nitrofurazone, furazolidone, furaltadone, and other antibiotics were selected for cross reactivity testing. The preparation of working solutions for all tested chemicals was similar to that for the NPAHD standard solution. The cross-reactivity values were calculated as follows: cross-reactivity = ([IC_{50} of NPAHD]/[IC_{50} of the competing compound]) × 100%. The IC_{50} value can be considered as a

measure (inverse) of the affinity of an antibody for a given analyte.

3. Results and discussion

3.1 Preparation and identification of immunogen

The molecular structure of haptens is very important to the preparation of antibodies. It is very difficult to prepare antibodies against micromolecule compounds that do not have complex groups, such as benzene rings, heterocycles, or branched structures. Such antibodies usually have very low titers. AHD is a small molecule hapten, so it needs to be coupled to a carrier protein to elicit a specific immune response. Reaction with 3-carboxybenzaldehyde (3-CBA) or 4-carboxybenzaldehyde (4-CBA) has been shown to be the most effective for the modification in the development of ELISA methods for determining levels of AHD [21, 25, 30]. In the present study, AHD was reacted with 4-carboxybenzaldehyle. The benzene ring and carboxyl group are key to preparing the antibody. Then the CPAHD was coupled with Jeffamine-BSA. The routes of synthesis are shown in Figure 1. The ^{1}HNMR spectroscopic data were as follows: ^{1}H NMR (DMSO, 20 °C δ vs. TMS, 400 M) δ 4.38 (s, 2H, CH_2), 7.80 (d, J = 8.4 Hz, 2H, ArH), 7.86 (s, 1H, N=CH), 8.00 (d, J =8.0 Hz, 2 H, ArH), 11.33 (s, 1 H, NH), 13.08 (s, 1H, COOH). Jeffamine-BSA, CPAHD, and CPAHD-Jeffamine-BSA were determined using UV spectroscopy. Results showed the maximum characteristic peaks to be 278 nm, 298 nm, and 286 nm, respectively, which indicated that CPAHD had been successfully coupled with Jeffamine-BSA. The structure of CPAHD was protected using Jeffamine as a spacer between CPAHD and BSA. In this way, the CPAHD molecule evoked a specific response against NPAHD as an antigenic entity. Jeffamine-BSA showed a higher linking capacity with CPAHD due to the greater numbers of primary amine groups available on Jeffamine-BSA than on BSA.

3.2 Preparation of monoclonal antibody and assay optimization

The indirect ELISA method was used to detect hybridoma monoclonal antibody production on the eighth day after cell fusion. The hybridomas that produced antibodies capable of recognizing NPAHD were subcloned three times using the limiting dilution method. The strain clones were passaged, frozen and subjected to several rounds of anabiosis. Then three hybridoma cells with a high inhibition ratio of OD_{450} (1B10, 3C3, and

3G10) were found to be stable. One of these hybridoma cell lines was used to prepare monoclonal antibody. The IC_{50} values of the three hybridomas detected by indirect ELISA (1B10, 3C3, and 3G10) were 4.15, 5.08, and 4.83 μg L^{-1}, respectively. The titers of the three hybridomas were 2.4×10^5, 1.6×10^5, and 1.6×10^5. According to the results, 1B10 was selected for the preparation of ascites. In dcCLELISA, the working concentrations of monoclonal antibody and CPAHD-HRP were 1:1000 and 1: 20,000 dilutions, respectively. In icCLELISA, the working concentrations of monoclonal antibody and coating antigen were 1:320,000 and 1:8000 dilutions, respectively. The recommended concentration of goat anti-mouse IgG-HRP was 1:4000 dilution. Under these conditions, the IC_{50} value was found to be 0.60 μg L^{-1} in both icCLELISA and dcCLELISA methodds. In a conventional immunoassay, indirect competitive ELISA was used to detect AHD [21, 25, 30] and the IC_{50} ranged from 0.68 to 3.8 μg L^{-1}. In the present study, the new methods were found to be more sensitive than the indirect competitive ELISA which was commonly used to detect AHD in animal tissues. The procedure required only 30 min in dcCLELISA, but icCLELISA required at least 1 h. The dcCLELISA method was found to be more suitable for screening AHD residues in food-producing animals. The substrates (Luminol, p-iodophenol, and urea peroxide) used in this experiment were optimized during a previous experiment and the results of this optimization are reported in another paper [31].

3.3 Assay validation

The LODs for dcCLELISA in fish and honey samples were 0.1 and 0.28 μg kg^{-1}, respectively. These values were all below the MRL (1 μg kg^{-1}) for residual AHD set by the European Commission. In this study, during the sample preparation the free AHD residues were reacted with 4-NBA into NPAHD to increase the molecular mass prior to detection. After the sample preparation, the NPAHD was detected using the dcCLELISA method. Finally, the concentration of NPAHD was determined and converted into AHD concentration according to the following formula $C_{AHD} = \frac{M_{AHD}}{M_{NPAHD}} \times C_{NPAHD}$. Where C_{NPAHD} is the concentration of NPAHD detected in the sample. M_{AHD} and M_{NPAHD} are the molecular weight of AHD and NPAHD, respectively. C_{AHD} is the concentration of

AHD calculated from the formula. As NPAHD is water-soluble and stable in PBS, the standard curve was established using NPAHD diluted in PBS, rather than a matrix matched calibration standard at concentrations of 0.03, 0.12, 0.5, 2.0, 8.0, 32.0 μg L^{-1}. The standard curve was shown in figure 2. The mean recovery and CV values of the two different tissues are outlined in Table 1. The samples were spiked with 0.25, 0.5, 2.5, and 10 μg kg^{-1} AHD, and the mean recovery values ranged from 83.6% to 94.7%, while CV values remained below 15%, both of which were within acceptable ranges [7,29].

3.4 Specificity of assay

The specificity of the CLELISA method was evaluated by determining the cross-reactivity toward NPAHD and AHD, CPAHD, AOZ, CPAOZ, NPAOZ, AMOZ, CPAMOZ, NPAMOZ, SEM, CPSEM, NPSEM, CP, NP, nitrofurantoin, nitrofurazone, furazolidone, furaltadone, ractopamine, clenbuterol, chloramphenicol and tetracycline. The cross reactivity values of CLELISA with NPAHD and with CPAHD were 100% and 39.67%, respectively. dcCLELISA showed no cross-reactivity (CR < 0.01%) with other compounds. The antibody showed excellent specificity and only showed significant cross-reactivity with CPAHD and NPAHD. The monoclonal antibody showed considerable cross-reactivity toward CPAHD as it is a modified hapten used to link to carrier protein Jeffamine-BSA in the preparation of immunogens. Antibodies linked to haptenic conjugates show a preferential recognition to the part of the molecule furthest from the site of attachment of the hapten to the carrier protein [32]. The structures of CPAHD and NPAHD were compared, and both were found to have moieties composed of the condensation of AHD and benzaldehyde. However, they showed different cross-reactivities. Considering that the substituents in the benzene ring are their only structural differences, it can be concluded that the phenyl moiety plays a certain role in antibody recognition despite its location close to the linking point of hapten and carrier protein.

3.5 Comparison of dcCLELISA,icCLELISA and a commercial kit in the detection of samples spiked with different concentrations of AHD

To demonstrate the applicability of CLELISA to the evaluation of levels of residual AHD in fish and honey samples, samples were simultaneously detected using dcCLELISA, icCLELISA, and commercial kits. As shown in Table 2, dcCLELISA and icCLELISA

showed a dynamic range of 0.03-32 μg L^{-1} with the lowest limit of detection (LOD) at 0.03 μg L^{-1}. In AHD detection, dcCLELISA was nearly 2-fold more sensitive than the conventional ELISA-based diagnostic kit. The correlations between dcCLELISA (X) and commercial kit (Y) are shown in figure 3. These results indicate the reliability of the ELISA for detecting residual levels of AHD in food samples.

4. Conclusions

In this study, a novel immunogen (CPAHD-Jaffermine-BSA) was synthesized and a monoclonal antibody with high specificity and sensitivity was prepared using this immunogen. In order to establish a highly sensitive enzyme-linked immunosorbent assay for screening AHD residuals in fish and honey, a dcCLELISA was developed to detect AHD. The dcCLELISA developed in this study was compared to a commercial kit in the assessment of fish and honey samples spiked with AHD at five different concentrations with good correlations. This method was nearly twice as sensitive as the conventional ELISA-based diagnostic kit and showed a broader detection range. It could be used for high-throughput monitoring of AHD in fish and honey samples and possibly other types of food.

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Table 1 Mean recovery rates of AHD from different kinds of samples using optimized dcCLELISA methods (n= 3)

Matrix	Concentration added (μg kg^{-1})	Recovery (%)	CV (%)
Fish	10	93.1	3.1
	2.5	89.0	5.5
	0.5	83.6	6.8
	0.25	86.0	4.9
Honey	10	94.7	3.6
	2.5	92.1	2.1
	0.5	88.2	3.5
	0.25	87.2	1.9

Table 2 Analytical comparison of dcCLELISA, icCLELISA, and a commercial kit in the detection of AHD

Analytical Parameters	dcCLELISA	icCLELISA	Commercially available ELISA kit.
Detection Range (μg L^{-1})	0.03-32	0.03-32	0.1-8.1
LOD (μg L^{-1})	0.03	0.05	Not provided
IC_{50} (μg L^{-1})	0.60	0.60	0.993
Time required (h)	0.5	1.5	0.75

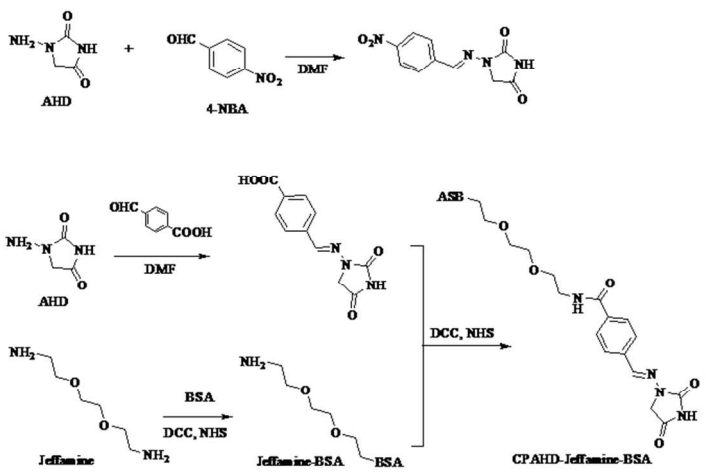

Fig. 1 Routes of synthesis of CPAHD, NPAHD, and CPAHD-Jeffamine-BSA. NPAHD was the target ananlyte derivative. CPAHD-Jeffamine-BSA was the immunogen.

254x190mm (96 x 96 DPI)

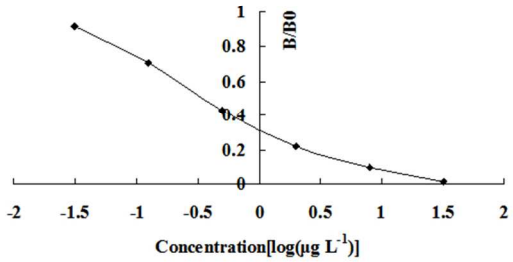

Fig. 2 Standard curve of optimized dcCLELISA. Each point represents the mean results of three well replicates. The "x" axis is the logarithm of the mean analyte concentration. The optical density of the B0 wells, containing all components except the competitor, was taken to represent 100%. The relative light unit (RLU) of the standard was normalized against the RLU of the zero standard (B/B0).

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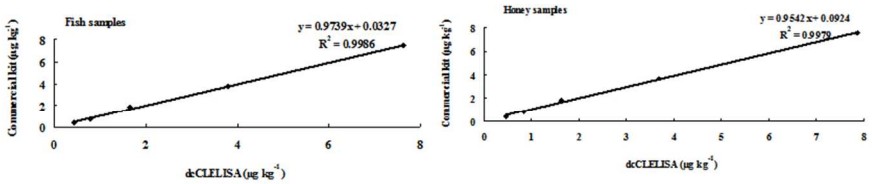

Fig. 3 Evaluations of dcCLELISA and the commercial kit. Each point represents the mean results of three spiked samples (fish or honey). The samples were detected respectively with the dcCLELISA and the commercial kit. The curve with the drug concentration in spiked sample obtained by the dcCLELISA method was plotted against the drug concentration in spiked sample obtained by the commercial kit.

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