Title: Development of monoclonal antibody-based ultrasensitive enzyme-linked immunosorbent assay and fluorescence-linked immunosorbent assay for 1-aminohydantoin detection in aquatic animals

Authors: Qi Sun, JinHua Luo, Lei Zhang, Zhihao Zhang, Tao Le

PII: S0731-7085(17)31325-0
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2017.06.068
Reference: PBA 11374

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 23-5-2017
Revised date: 28-6-2017
Accepted date: 30-6-2017

Please cite this article as: Qi Sun, JinHua Luo, Lei Zhang, Zhihao Zhang, Tao Le, Development of monoclonal antibody-based ultrasensitive enzyme-linked immunosorbent assay and fluorescence-linked immunosorbent assay for 1-aminohydantoin detection in aquatic animals, Journal of Pharmaceutical and Biomedical Analysishttp://dx.doi.org/10.1016/j.jpba.2017.06.068

Development of monoclonal antibody-based ultrasensitive enzyme-linked immunosorbent assay and fluorescence-linked immunosorbent assay for 1-aminohydantoin detection in aquatic animals

Qi Sun [a], JinHua Luo [b1], Lei Zhang [a], Zhihao Zhang [a], Tao Le [a *]

[a] *College of Life Science, Chongqing Normal University, Chongqing 401331, P. R. China*

[b] *Chongqing Institute of Biotechnology, Chongqing 401123, P. R. China*

[*] Corresponding author. Tel.: +86 23 67301531; fax: +86 23 67301531; *E-mail address*: hnxylt@163.com.

[1] Co-first author.

Highlights

1. This is the first report of QDs-FLISA to detect AHD developed based on a specific monoclonal antibody.

2. The FLISA offers higher sensitivity in comparison with ic-ELISA.

3. Excellent correlations of the ic-ELISA/LC-MS/MS and FLISA/LC-MS/MS data were observed for processed samples.

Abstract

Monitoring and rapid evaluation of nitrofurantoin metabolite, 1-aminohydantoin (AHD)，are important for food safety and human health. Herein, we established the monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) and quantum dots (QDs) -fabricated fluorescence-linked immunosorbent assay (FLISA). Monoclonal antibody specific to nitrophenyl derivative of AHD was derived from hybridoma cell lines 3.2.4/5A8. For another, CdTe core QDs with emission wavelength of 605 nm were also synthesized. The performances of the proposed ic-ELISA and FLISA were further examined and the corresponding results were also validated by standard LC-MS/MS analysis. The obtained results indicated that both ic-ELISA and FLISA exhibited good dynamic linear detection for NPAHD over the range from 0.1 to 3.0 ng mL^{-1}. Meanwhile, proposed immunosorbent assays are characterized by satisfactory recovery rates of 81.5-113.7%. The experimental data suggested these two immunoassays could be facile, cost-effective and rapid tools for the prospective quantitative method for AHD analysis in food matrix.

Keywords: Nitrofurantoin metabolite; 1-aminohydantoin; Enzyme-linked immunosorbent assay; Fluorescence-linked immunosorbent assay; Aquatic animals

1. Introduction

Nitrofurantoin containing a characteristic 5-nitrofuran ring group is a typical representative of the family of nitrofuran drugs. As a synthetic broad-spectrum antibiotic, nitrofurantoin was often used extensively both as active ingredient in the treatment of gastrointestinal infections and as growth promoter in the feeding of food-producing animals [1-3]. Most of nitro furan compounds, in particular nitrofurantoin, are thought to be mutagenic as well as carcinogenic, the residue of which is one of the most critical health problems. Nevertheless, there is still illegal abuse of nitro furan compounds due to their low cost and high benefit in some countries and regions [4], which is closely related to food safety and human health. Thus accordingly, human health agencies around the world have not only listed nitrofurantoin and other nitro furan compounds as prohibited drugs in animal husbandry [1, 5], but strictly regulated their legal permissible level in food-producing animals [4, 6]. For example, Europe March 13, 2003 decisions had set the minimum requirement performance limit (MRPL) at 1 μg kg^{-1} for each nitrofuran metabolite residue in poultry meat and aquaculture products [7]. Hence, the efficiency of both rapid and precise evaluation of nitrofurantoin is of great importance. However, direct determination of the above mentioned compound is almost impossible to achieve because it is highly unstable and rapidly excreted *in vivo*. No parent drug can be detected in tissues after withdrawal of medication in animals. As illustrated in Fig. 1, 1-aminohydantoin (AHD) is a metabolite of nitrofurantoin which has been recognized as a marker residue for evaluation of nitrofurantoin in food-producing animals [8]. Under normal circumstances, AHD is covalently bound to tissue proteins, and must be released from the

tissues under acidic conditions and derivatized with *o*-nitrobenzaldehyde to form the nitrophenyl-AHD (NPAHD), which is a suitable structure for detection [9].

There have been diverse analytical methods developed for the determination of AHD, for example, liquid chromatography-electrospray ionization tandem mass spectrometry, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) [10-18]. The above mentioned instrumental approaches are widely accepted by many reference laboratories owing to their high sensitivity and satisfactory accuracy. However, expensive instruments, time-consuming preparation steps are usually needed for these assays. Therefore, the laboratory-based techniques may not meet the requirements for the practical applications in a wide variety of fields. It is necessary to develop measurements which are characterized by simple operation, low cost as well as high accuracy.

Immunochemical method, by contrast, is widely drawing considerable attention due to its low cost, fast analysis, excellent feasibility and high specificity, which make it particularly useful in routine monitoring of food matrix. Recently, using antibodies specific to a derivate of AHD, an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has also been employed for the determination of AHD; however, ELISA method lacks stability because of the use of enzyme as a biomarker [4, 19-21]. On the other hand, quantum dots (QDs) have attracted interest of the biosensing community due to their unique luminescent properties. Compared to conventional fluorophores, QDs are regarded as a promising reporters for immunoassay because of their excellent fluorescent properties, such as size-tunable fluorescence, high quantum yields, broad adsorption spectra, narrow and symmetric photoluminescence spectra, strong

luminescence, and high resistance to photo-bleaching [22-24]. Since QDs possess excellent and promising luminescent properties, they have been utilized for the sensitive screening of small molecule chemicals based on the development of QDs-fabricated fluorescence-linked immunosorbent assay (FLISA) as shown in our previous works [25, 26]. The excellent application of quantum dots could greatly improve the detection ability of targets. To the best of our knowledge, however, there has been no report yet on the application of QDs as the fluorescence probe for the determination of AHD. Herein, we established a hybridoma clone as the source of a monoclonal antibody (mAb) that is specific to derivate of AHD and then applied it to detect AHD with the development of immunosorbent assays. The performance of the proposed ic-ELISA and FLISA were examined in this study and the corresponding determination results were validated by LC-MS/MS as a reference method.

2. Materials and methods

2.1. Chemicals and materials

Nitrofurantoin, nitrofurazone, furazolidone, furaltadone, AHD, nitrophenyl-AHD (NPAHD), 2-nitrobenzaldehyde (2-NBA), 4-carboxybenzaldehyle (4-CBA), Freund's complete and incomplete adjuvants, bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma-Aldrich (St. Louis, MO). Peroxidase-labeled goat anti-mouse IgG (IgG-HRP) was supplied by Sino-American Biotechnology (Shanghai, Branch, China). *N*,*N*-dicyclohexylcarbodiimide, *N*,*N*-dimethylformamide, *N*-hydroxysuccinimide, tween 20, methanol, dimethyl sulfoxide and ethyl acetate were obtained from Sinopharm

Chemical Reagent Co. Ltd (Beijing, China).

2.2. Synthesis of haptens

As demonstrated in Fig.2, carboxyphenyl derivative of AHD (CPAHD) is obtained by the reaction between AHD and 4-CBA. 1.5 mL of DMF was added to 0.65 g of 4-CBA in 6 mL of methanol with stirring. Then AHD (0.45 g) was added to the reaction mixture and refluxed for overnight at 65 °C under stirring. Formation of the target products were monitored by thin-layer chromatography. After the reaction was over according to thin-layer chromatography, the reaction mixture was filtered and washed with ethanol several times to remove any unreacted 4-CBA. The CPAHD conjugate was stored at 4 °C for the following experiment.

2.3. Preparation of immunogen and coating antigens

Synthetic routes to the derivatives and conjugates are shown in Fig.2. Briefly, 12.4 mg of CPAHD, 11.6 mg of *N*-hydroxysuccinimide and 20.6 mg of *N*,*N*-dicyclohexylcarbodiimide were dissolved in 500 μL of *N*,*N*-dimethylformamide. Then the reaction mixture was stirred gently overnight at 4 °C and centrifuged at 2500 rpm for 10 min. Subsequently, the obtained supernatant was added dropwise to 90 mg of BSA or 60 mg of OVA dissolved in 9.5 mL of phosphate buffered solution (PBS, pH 7.4) under stirring. The conjugates were stirred at 4 °C for 12 h and purified on Sephadex G-25. The eluted conjugates were then dialyzed against PBS (0.01 M, pH 7.4) at 4 °C for 3 days and then stored at 4 °C. The formed CPAMOZ-BSA and CPAMOZ-OVA were used as immunogens and coating antigens, respectively.

2.4. Production of mAb

All animal experiments in this study adhered to the Chongqing Normal University animal experiment center guidelines and were approved by the Animal Ethics Committee (CQNU-2015-02-003). Eight BALB/c female mice (6-8 weeks old, supplied by the Chongqing Center for Disease Control and Prevention) were immunised with CPAHD-BSA conjugates. The first dose consisted of 50 μg of CPAHD-BSA that was intraperitoneally injected in an emulsion of PBS and complete Freund's adjuvant (1:1, v/v). It was then injected subcutaneously into multiple sites on the back of each mouse. After three booster doses of CPAHD-BSA (50 μg) in incomplete Freund's adjuvant at 2-week intervals, the antiserum titer was determined by a non-competitive indirect ELISA. Three days before cell fusion, the mouse exhibiting the highest titer and specificity was immunized with a final dose of CPAHD-BSA (100 μg) in distilled PBS.

Cell fusion procedures were performed as previously described with some modifications [26]. Hybridoma cell lines were produced through the fusion of myeloma cells (Sp2/0) at a ratio of 5-10:1, and spleen cells obtained from immunized mice. Hybridoma from wells having a positive response in the ELISA described below were cloned three by limiting dilution method. Stable antibody-producing clones were expanded until monoclones were obtained. After cell culture, the hybridoma cell was intraperitoneally injected into paraffin-primed mice to produce ascitic fluids. Ascitic fluids were collected purified by the caprylic acid and ammonium sulfate precipitation method followed using a protein G affinity column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. The purified mAb was stored at -20 ºC

before use.

2.5. The procedures of ic-ELISA

The protocol of ic-ELISA was carried out using the methodology described previously with a slight modification [26]. Maxisorp 96-well microtiter plates were coated with CPAHD-OVA (200 μg L^{-1}) in 100 μl of coating buffer (0.05 mol L^{-1} carbonate buffers, pH 9.6) overnight at 4 ℃. After the plates were washed three times with PBS containing 0.1% Tween-20 (PBST), and the excess binding sites were blocked with 5% glycine and 5% sucrose in PBS (250 μL per well) for 3 h at 37 ℃. Then, the plates were washed with PBST, and then NPAHD (as the competitor) was serially diluted in PBS at different concentrations or sample extracts were added at 50 μL per well, and 50 μL per well of the ant-NPAHD antibody was added to a microtiter plate. After incubation at 37 ℃ for 30 min, the plates were washed with PBST, and then 100 μL of HRP-IgG (1:5000) were added and incubated at 37 ℃ for 30 min. After washing the plate with PBST, 100 μL of the TMB substrate solution were added to each well and incubated for 15 min at 37℃ in the dark. Then, the reaction was stopped with 50 μL of 2 M H_2SO_4. The absorbance was measured at 450 nm using a Tecan Sunrise 2.5 Microplate Reader (SUNRISE, Austria). A calibration curve was generated in the form $(B/B_0) \times 100\%$ vs. log C, where B and B_0 were the absorbance of the analyte at the standard point and at zero concentration of the analyte, respectively. The 50% inhibition concentration (IC_{50}) was determined as a measure of the sensitivity of the ic-ELISA [3]. The limit of detection (LOD) was based on 20 blank samples accepting no false positive rates.

2.6. Determination of cross-reactivity

The extent of cross-reactivity (CR) was evaluated by determining IC_{50} values using the ic-ELISA described above. Several nitrofuran analogues (nitrofurantoin, furazolidone, furaltadone, and nitrofurazone) and their metabolites (1-aminohydantoin (AHD), 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethy l-2-oxazolidinone (AMOZ), and semicarbazide (SEM), nitrophenyl derivates (NPAHD, NPAOZ, NPAMOZ, and NPSEM), carboxyphenyl derivative (CPAHD, CPAOZ, CPAMOZ, and CPSEM), and derivatizing reagents were selected for testing CR (Table 1). The concentrations of the above compounds covered the range from 0.01 to 1,000 ng mL^{-1}. CR values were calculated as follows: % CR = [(IC_{50} value of NPAHD) / (IC_{50} value of other analytes)] × 100%. The mAb with the lowest IC_{50} for NPAHD was selected for the remainder of this study.

2.7. Synthesis of CdTe core QDs

CdTe/CdSe core/shell nanostructure QDs with emission at 605 nm were synthesized according to a previously reported method [27]. Briefly, 182 mg of $CdCl_2 \cdot 2.5H_2O$ was dissolved in 40 mL of toluene, ultrapure water in a round bottom flask, and 240 mg glutathione, 10 mg trisodium citrate dehydrate, 2 mL of Na_2TeO_3 and 20 mg of $NaBH_4$ were added into cadmium sol with constant stirring. The pH was adjusted to 10.5 with 1 M of NaOH. The mixtures were kept in ultra-sonicator for vigorous stirring at room temperature for 30 min. All reactions were carried out under ambient atmospheric conditions. The as-obtained mixture was prepared at 600 W under microwave irradiation.

Mixture reaction time increased with the time interval of 20 s. Series of high-quality CdTe QDs was cooled down to 50 °C. The CdTe QDs were precipitated using 2-propanol

and the solution was centrifuged at 4,000 rpm for 5 min. The CdTe QDs dispersion was resuspended in PBS buffer at pH 7.4. The CdTe QDs were characterised by the Unicam UV500 UV–vis spectrophotometer (Thermo Spectronic, USA) and luminescence spectrometer LS-55 (Perkin-Elmer, Inc., Waltham,MA).

2.8. Preparation of mAb-QDs conjugates

The formation of antibody-conjugated QDs was synthesized using the activated ester approach. Briefly, 0.1 mL of QDs diluted in 1 mL of reaction buffer (10 mM sodium borate, pH7.4), was pre-activated with 0.05 mL of a freshly prepared aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (10 mg mL^{-1}) for 20 min at room temperature. Then, 0.2 mg of anti-NPAHD mAbs were added to the carboxyl-activated QDs and incubated for 30 min at room temperature. Then, 200 mL *N*-hydroxysulfosuccinimide solution was added and the same mixture was reacted at 4 °C for a further overnight incubation. The resulting mixture was centrifuged three times at 14,000 rpm to remove any unbound QD labeled antibodies. Then the precipitates were resuspended with 10% Triton X-100. The conjugated bio-complexes were characterized by UV-vis absorption and luminescence spectrometer, and then stored at 4 °C.

2.9. Competitive indirect FLISA procedure

The same pair of immunoreagents, CPAHD-OVA, were selected to develop the FLISA for AHD determination. The protocol used for the competitive indirect FLISA was similar to our previously described procedures with some modifications [25]. The checkerboard procedure was used to optimize the coating antigen and the primary antibody

concentrations. To each well of 96-well maxisorp microtiter plate was added 100 µl of 200 µg L^{-1} of CPAHD-OVA solution in 0.05 M $NaHCO_3$ (pH 9.6), and incubation was performed overnight at 4 °C. The plates were washed three times with PBST and then incubated (37 °C) with 200 µL per well of 0.1% gelatin in PBS for 2 h. After the blocking solution was removed, NPAHD (as the competitor) at different concentrations, or 50 µL of test sample extract, were then pipetted into the coated microtiter well and then 50 µL each of mAb-QDs were added into each well. After incubation (37 °C) for 1 h, the plates were washed three times with PBST. Then the fluorescence intensity was recorded by SpectraMax M2e microplate reader (Molecular Devices, USA). Using an excitation wavelength of 335 nm and emission wavelengths of 605 nm, standard curves were plotted from which IC_{50} values and LODs for NPAHD were obtained.

2.10. Samples preparation

The tissue samples were minced, homogenized and fortified with AHD at the content of 0.06, 0.15, 0.6 and 1.5 µg kg^{-1}. The samples were then derivatized and extracted according to the procedure described in the literature. In brief, every fortified sample (1.0 g each) was weighed into 50 mL polypropylene centrifuge tubes, then 3 mL methanol was added. The mixtures were vortexed for 10 s at room temperature, and were processed for 10 min in a boiling water bath. 4 mL deionized water, 0.5 mL 1 M HCl, and 100 µL 2-NBA (50 mM) in dimethyl sulfoxide were added to the mixtures. Samples were vortexed for 30 s and incubated at 37°C overnight. Then the samples were extracted by mixing with 5 mL of 0.1 M K_2HPO_4 , 0.4 mL of 1 M NaOH , and 5 mL of ethyl acetate, followed by centrifugation at 5000 rpm at 4 °C for 20 min. The

ethyl acetate fraction was collected and dried under a stream of nitrogen. Then the residue was re-dissolved in 300 μL of PBS. The remaining extracts were simultaneously analyzed by ic-ELISA and FLISA. It was noticed that for 1 g of the sample fortified with AHD at the content of 0.06, 0.15, 0.6 and 1.5 μg kg^{-1}, after pretreatment and substituting in 300 μL of PBS, the final concentration of AHD in the extracted solutions was theoretically 0.2, 0.5, 2.0 and 5 ng mL^{-1}.

2.11. Validation of ic-ELISA and FLISA

Validation of ic-ELISA and FLISA was carried out using 20 different samples including in catfish, carp and shrimp muscle purchased from local markets. The samples were previously confirmed using LC-MS/MS analysis, to be free of AHD compounds. LOD determination was based on 20 blank samples, accepting no false positive rates, with an average +3 standard deviation (SD). The accuracy and precision of the method were represented by the recovery and coefficient of variation (CV), respectively. Recoveries (%) of fortified AHD were determined using five fortified duplicate blanks at 0.2, 0.5, 2.0 and 5 μg kg^{-1} in four different analyses. The recovery (%) was calculated by the following equation: (conc. measured/conc. fortified) × 100. CVs were determined from the analysis of the above samples. Analysis of each concentration level was repeated in quintuplicate over a time span of two months.

2.12. Comparison of ic-ELISA, FLISA and LC-MS/MS

In order to demonstrate the applicability of the ic-ELISA and FLISA for real sample analysis, the proposed methods were used for the determination of AHD residue in some

food samples of seventy unknown samples, including ten carp, six grass carp, nine crucian carp, five chub, five catfish, six shrimp, eight chicken muscle, seven swine muscle, nine milk, and five cattle muscle purchased from local markets and supermarkets. The content of AHD in the above samples was detected by ic-ELISA and FLISA method. At the same time, the LC-MS/MS was used as a reference method which was offered by EU [13].

3. Results and discussion

3.1. Preparation and identification of immunogen

It is well known that the hapten design of the corresponding immunogen is critical to the identification of the target analyte by the specific antibody. As small molecule compounds, both nitrofurantoin and AHD are firstly needed to bind to carrier proteins in order to render them highly immunogenic. As described above, direct determination of nitrofurantoin is almost unrealistic owing to its being extremely unstable and rapidly excreted *in vivo*. By contrast, AHD, the metabolite of the target molecule, could be bound to tissue proteins and persist for considerable period of time in tissues. Therefore, AHD is treated as the target marker for the detection of nitrofurantoin. In order to prevent its rebinding, AHD is firstly needed to be reacted with some derivative reagents. In general, the derivative form of AHD is NPAHD, but its structure is not suitable for the preparation of the immunogen. On the other hand, it is reported that AHD could be reacted with carboxybenzaldehyle (3-CBA or 4-CBA) in order to form immunizing haptens [4, 19, 20]. Under this circumstance, AHD was derivatized with the attachment of a carboxyphenyl

moiety to form CPAHD whose structure is similar to NPAHD. Therefore, AHD was reacted with 4-carboxybenzaldehyle to form CPAHD so that we could obtain a desirable hapten mimicking the NPAHD derivative, which was a key step in antibody production and illustrated in Fig.2. CPAHD could be conjugated with BSA by the carboxylic acid spacer to form immunogens. It should be noted that both the benzene ring and the carboxyl group are critical to the preparation of the mAb. As depicted in Fig.3, UV-VIS spectroscopy results demonstrated the maximum absorption wavelength to be 310 nm, 278 nm and 313 nm for CPAHD, BSA, and CPAHD-BSA, respectively. Moreover, the above results also indicated that carrier protein BSA has been successfully conjugated to CPAHD and CPAHD-BSA could be used in following animal immunization experiment.

3.2. Characterization of the monoclonal antibody

After immunization of mice, the value of antiserum titer for mAb against NPAHD was calculated to be 32,000 by the indirect ELISA assay. Hybridomas cells (hereinafter referred to as 3.2.4/5A8), which may produce stable and specific mAb against NPAHD, could be obtained after the operation of cell fusion and cloning. In order to evaluate mAb sensitivity, the binding and competitive properties of the antibody were examined by ic-ELISA assay under the optimal conditions. The value of antiserum titers was 2.56×10^4 for these two hybridomas. Meanwhile, the IC_{50} value of mAb (3.2.4/5A8) was also evaluated to be as low as 0.57 ng mL^{-1} for NPAHD. Furthermore, IC_{50} values and cross-reactivities of selected compounds tested by ic-ELISA are shown in Table 1. It could be inferred that immune response of mAb (3.2.4/5A8) presented in this work is characterized by a high specificity as well as a good sensitivity when compared with those

reported antibodies previously [4, 19-21].

3.3. Characterizations of CdTe QDs and mAb-QD conjugates

The morphology and optical properties of QDs were characterized by high-resolution transmission electron microscopy and spectrophotometer approaches, respectively. As shown in Fig.4 (A), prepared QDs are almost uniformly distributed in spherical shape with the average size of 62 nm. The results for optical properties of QDs and mAb-QD were also depicted in Fig.4 (B). On one hand, these QDs in our study were characterized by broad excitation spectra and narrow emission spectra. On the other hand, CdTe QDs displayed absorption and emission peaks centered at 555 nm and 605 nm, respectively as illustrated in Fig.4 (B). The absorption and corresponding emission maximum wavelengths of QDs are greatly blue shifted which could be attributed to the size effect of nanoparticles. The full width at half maximum of QD was 35 nm with high fluorescent quantum yield around 50-65%. Furthermore, the fluorescence intensity and the peak wavelength of CdTe QD and mAb-QD conjugates were also detected by fluorescence spectrometer method. The obtained result suggested that the mAb-QD conjugates have the same emission peaks centered at 605 nm as QDs (Fig.4 (B)). The full width at half maximum of QD and mAb-QD conjugates did not change. It could be inferred that there is no reunion phenomenon occurred during the course of QDs conjugating to antibody. Based on the above spectra results, we may conclude that QD conjugates to the specific antibody were effective. The fluorescent area of mAb-QD was smaller than QD and the interference of QDs emission wavelength and fluorescence intensity by mAb-QD conjugates is almost negligible. So, it is possible to develop a FLISH assay with the above

mentioned tracer.

3.4. Sensitivity of the ic-ELISA and FLISA

In this study, both ic-ELISA and FLISA method were established for the detection of AHD residue. The AHD residue was derivatized into NPAHD for the detection by the developed ic-ELISA and FLISA methods. The standard solutions of NPAHD were diluted in PBS to produce the concentration range from 0.01 to 100 ng mL^{-1}. The standard curve was established using NPAHD diluted in PBS, rather than a matrix matched calibration standard, because NPAHD was water-soluble and stable in PBS. The sensitivities of the ic-ELISA and FLISA methods were evaluated using the values of IC_{50} and LOD, which were obtained from the standard curves referred by NPAHD molecule. The competitive inhibition between anti-NPAHD mAbs and CPAHD-OVA was demonstrated in Fig.5 (A) which was evaluated by increasing NPAHD concentrations. It is clearly noted that B/B_0 value decreased linearly with the rise of the logarithm level of NPAHD over the range from 0.1 to 3.0 ng mL^{-1} (Fig.5 (B)) with respect to ic-ELISA assay. The corresponding IC_{50} and LOD values were calculated to be 0.57 ng mL^{-1} and 0.13 ng mL^{-1}. Specific features of FLISA assay are also demonstrated here. IC_{50} value for NPAHD is 0.44 ng mL^{-1} and the detection limit is 0.09 ng mL^{-1}. The concentration in the range of 0.1 to 3.0 ng mL^{-1} with acceptable correlation coefficients (R^2=0.9915) is also obtained here. By comparing the results obtained by both immunoassays with different detection modes, it could be revealed that FLISA method possesses a higher sensitivity than ic-ELISA assay. In addition, the detection limit and the dynamic range of the as-developed immunoassays can meet the requirement for monitoring AHD residue in foodstuffs.

3.5. Validation of ic-ELISA and FLISA

The animal tissue samples (including catfish, carp and shrimp) collected from local retail stores, were analyzed by ic-ELISA and FLISA. The sample preparation of the ic-ELISA and FLISA method included the following procedures: acid hydrolysis, derivatization, extraction, enrichment, clean up. The developed ic-ELISA and FLISA method involved acid hydrolysis for the release of tissue bound residues into the solution. During the sample preparation, the free AHD residues were reacted with 2-NBA into NPAHD to increase the molecular mass prior to detection. After the extraction, enrichment and clean up procedures, the NPAHD was detected using the ELISA and FLISA method. Finally, the concentration of NPAHD was determined and converted into AHD concentration according to the following formula: Concentration of AHD = (molecular weight of AHD/molecular weight of CPAHD) × concentration of CPAHD. The matrix effects are one of the most common challenges in performing immunoassays on complex samples. In this study, to evaluate the influence of the matrix on the ic-ELISA and FLISA, animal tissue samples were diluted with PBS, and the schemes of dilution are shown in Table 2. The LODs of the ic-ELISA and FLISA for NPAHD in catfish, carp and shrimp samples were 0.13-0.22 μg kg^{-1} and 0.09-0.14 μg kg^{-1}, respectively, which corresponded to 0.06-0.10 μg kg^{-1} and 0.04-0.06 of AHD after calculation using the above formula. The LODs for for the three above mentioned animal tissues were below 0.10 μg kg^{-1}, which is lower than the MRPL for AHD residue set by the EU. Recoveries of AHD for both ic-ELISA and FLISA assay were determined and the results are presented in Table 2. For ic-ELISA analysis, the recoveries ranged from 81.5% to 113.7% and the CVs were less

than 11.8% in various biological matrices. Under the same experimental conditions, the recoveries ranged from 82.4% to 107.6% with the CVs less than 10.6% for FLISA assay. It could be indicated that the matrix has no significant effect on the sensitivity of the ic-ELISA and FLISA followed by the extraction method used in this study. In general, the commonly accepted recovery value for an ic-ELISA is between 80 and 120% [19]. Percentage recovery tests exhibited excellent recoveries of AHD for proposed immunological methods (Table 2), indicating that the accuracy and precision of both proposed assays are within an acceptable range.

3.6. Comparison of ELISA, FLISA and LC-MS/MS method

The analytical performances of the ic-ELISA and FLISA were validated by determining AHD levels in animal tissue samples. Meanwhile, the concentrations of AHD in samples were detected following the standard LC-MS/MS method. As shown in Table 3, AHD was not detected in all studied samples obtained from local markets and supermarkets. These results suggested that the present ic-ELISA and FLISA are reliable for the detection of AHD in edible tissues of animals.

4. Conclusions

The present study reports the production and characterisation of a specificity mAb (3.2.4/5A8) against NPAHD, which showed the cross-reactivity to CPAHD (74.0%) and AHD (14.9%). The mAb can be successfully labeled with CdTe QDs by covalent binding using EDC. The aim of the present study was to develop rapid, simple, accurate and sensitive analytical method for the determination of AHD. With respect to two different

detection modes, recoveries of 81.5-113.7% were obtained by spiking with AHD blank animal tissue samples with CVs below 15%. Moreover, the ic-ELISA and FLISA were also validated by LC-MS/MS with good correlation. The results obtained here suggested these two immunoassays could be considered as feasible quantitative assays for AHD evaluation in food-originating animals.

Disclosure statement

The authors declare no competing financial interest.

Acknowledgments

This project was supported by the Scientific and Technological Research Project of Chongqing China (Project No. cstc2016shmszx80069); China Scholarship Council (CSC No. 201608505051); the National Natural Science Foundation of China (Grant No. 31671939) and Chongqing Normal University.

Appendix A. Supplementary materials

Routes of synthesis of CPAHD, NPAHD, CPAHD-BSA, and CPAHD-OVA; Ultraviolet wavelength scanning of CPAHD, BSA and CPAHD-BSA.

References

[1] Y. Tang, X. Xu, X. Liu, X. Huang, Y. Chen, W. Wang, J. Xiang, Development of a lateral flow immunoassay (LFA) strip for the rapid detection of 1-aminohydantoin in meat samples, J. Food Sci. 76 (2011) T138-143.

[2] Y.P. Xu, L.Q. Liu, Q.S. Li, C.F. Peng, W. Chen, C.L. Xu, Development of an immunochromatographic assay for rapid detection of 1-Aminohydantoin in urine specimens, Biomed. Chromatogr. 23 (2009) 308-314.

[3] T. Le, H. Yu, Determination of 3-Amino-2-oxazolidinone in animal Tissue by an Enzyme-Linked immunosorbent assay and a time-resolved fluoroimmunoassay, Anal. Lett. 48 (2015) 1275-1284.

[4] W. Liu, C. Zhao, Y. Zhang, S. Lu, J. Liu, R. Xi, 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, J. Agric. Food. Chem. 55 (2007) 6829-6834.

[5] G.R. Stehly, S.M. Plakas, K.R. el Said, Liquid chromatographic determination of furazolidone in shrimp, J. AOAC Int. 77 (1994) 901-904.

[6] EC, Commission regulation (EC) 1442/95, Official Journal of the European Community. L143 (2005) 26.

[7] EC, Commission decision 2003/181/EC 13 March 2003, amending decision 2002/657/EC as regards the setting of minimum performance limits (MRPLs) for certain residues in food animal origin., Official Journal of the European Community. L71 (2003) 17.

[8] W. Jin, G. Yang, H. Shao, A. Qin, A label-free impedimetric immunosensor for detection of 1-aminohydantoin residue in food samples based on sol-gel embedding antibody, Food Control 39 (2014) 185-191.

[9] I. Diblikova, K.M. Cooper, D.G. Kennedy, M. Franek, Monoclonal antibody-based ELISA for the quantification of nitrofuran metabolite 3-amino-2-oxazolidinone in tissues using a simplified sample preparation, Anal. Chim. Acta 540 (2005) 285-292.

[10] L. Saari, K. Peltonen, Novel source of semicarbazide: levels of semicarbazide in cooked crayfish

samples determined by LC/MS/MS, Food Addit. Contam. 21 (2004) 825-832.

[11] S.V. de Souza, R.G. Junqueira, R. Ginn, Analysis of semicarbazide in baby food by liquid chromatography tandem mass spectrometry (LC-MS-MS)--in-house method validation, J. Chromatogr. A 1077 (2005) 151-158.

[12] Y. Zhang, H. Qiao, C. Chen, Z. Wang, X. Xia, Determination of nitrofurans metabolites residues in aquatic products by ultra-performance liquid chromatography-tandem mass spectrometry, Food Chem. 192 (2016) 612-617.

[13] D. Wilasinee, P. Sutthivaiyakit, S. Sutthivaiyakit, Determination of nitrofurans in chicken feed by high-performance liquid chromatography–tandem mass spectrometry, Anal. Lett. 48 (2015).

[14] N.M. Valera-Tarifa, P. Plaza-Bolaños, R. Romero-González, J.L. Martínez-Vidal, A. Garrido-Frenich, Determination of nitrofuran metabolites in seafood by ultrahigh performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry, J. Food Compos. Anal. 30 (2013) 86-93.

[15] D. Kim, B. Kim, S.W. Hyung, C.H. Lee, J. Kim, An optimized method for the accurate determination of nitrofurans in chicken meat using isotope dilution–liquid chromatography/mass spectrometry, J. Food Compos. Anal. 40 (2015) 24-31.

[16] A. Radovnikovic, M. Moloney, P. Byrne, M. Danaher, Detection of banned nitrofuran metabolites in animal plasma samples using UHPLC–MS/MS, J. Chromatogr. B 879 (2011) 159–166.

[17] A.S. Pereira, J.L. Donato, G. De Nucci, Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products, Food addit. Contam. 21 (2004) 63-69.

[18] G.O. Noonan, C.R. Warner, W. Hsu, T.H. Begley, G.A. Perfetti, G.W. Diachenko, The determination of semicarbazide (N-aminourea) in commercial bread products by liquid chromatography-mass spectrometry, J. Agric. Food. Chem. 53 (2005) 4680-4685.

[19] U. Chadseesuwan, S. Puthong, O. Gajanandana, T. Palaga, K. Komolpis, Development of an enzyme-linked immunosorbent assay for 1-aminohydantoin detection, J. AOAC Int. 96 (2013) 680-686.

[20] Q. Wang, Y. Liu, Y. Chen, W. Jiang, J. Shi, Y. Xiao, M. Zhang, Development of a direct competitive chemiluminescent ELISA for the detection of nitrofurantoin metabolite 1-amino-hydantoin in fish and honey, Anal. Methods. 6 (2014) 4414-4420.

[21] W.X. Jiang, P.J. Luo, X. Wang, X. Chen, Y.F. Zhao, W. Shi, X.P. Wu, Y.N. Wu, J.Z. Shen, Development of an enzyme-linked immunosorbent assay for the detection of nitrofurantoin metabolite, 1-amino-hydantoin, in animal tissues, Food Control. 23 (2012) 20-25.

[22] T. Le, Y. Xie, L.Q. Zhu, L. Zhang, Rapid and sensitive detection of 3-amino-2-oxazolidinone using a quantum dot-based immunochromatographic fluorescent biosensor, J. Agric. Food. Chem. 64 (2016) 8678-8683.

[23] F. Di Nardo, L. Di Nardo, C. Giovannoli, C. Passini, V.V. Goftman, I.Y. Goryacheva, C. Baggiani, A fluorescent immunochromatographic strip test using Quantum Dots for fumonisins detection, Talanta 150 (2016) 463–468.

[24] Y. Xie, L. Zhang, X. Yang, T. Le, Development of a quantum dot-based immunochromatography test strip for rapid screening of oxytetracycline and 4-epioxytetracycline in edible animal tissues, Food Addit. Contam. A 34 (2017) 371–378.

[25] T. Le, L. Zhu, H. Yu, Dual-label quantum dot-based immunoassay for simultaneous determination of Carbadox and Olaquindox metabolites in animal tissues, Food Chem. 199 (2016) 70-74.

[26] T. Le, L. Zhu, X. Yang, A quantum dot-based immunoassay for screening of tylosin and tilmicosin in edible animal tissues, Food Addit. Contamin. A 32 (2015) 719-724.

[27] P. Bichpuria, A. Oudhia, Synthesis and optical studies of CdTe/CdSe core/shell nanostructure, Int. J. Lumin. Appl. 1 (2016) 101-103.

Figure Captions

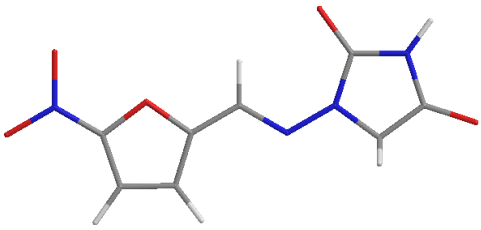
Fig. 1. The mojiangcular structures of nitrofurantoin, AHD, CPAHD, NPAHD, 4-CBA and 2-NBA.

Fig. 2. Routes of synthesis of CPAHD, NPAHD, CPAHD-BSA, and CPAHD-OVA. CPAHD and NPAHD were respectively derived with 4-CBA and 2-NBA from AHD. CPAHDZ was used to synthesize the immunogen and coating antigen, while NPAHD was the target sample derivative.

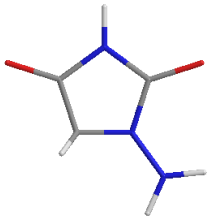
Fig. 3. Ultraviolet wavelength scanning of BSA, CPAHD and CPAHD-BSA.

Fig. 4. Characterization of the structural and optical properties of QD. (A) the HRTEM of QDs; (B) UV-vis absorption and fluorescence spectra curve of QD and mAb-QD. The emission peak is at 605 nm, with full width at half-maximum of 35 nm.

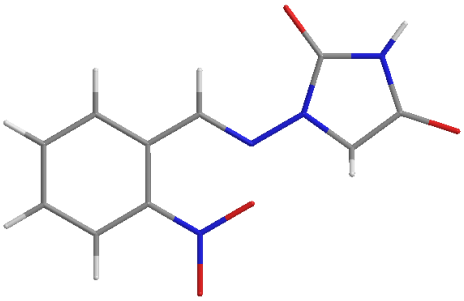
Fig. 5. The standard curve for NPAHD using ic-ELISA and FLISA. B and B_0 are the absorbances of the sample with/without NPAHD, respectively. Each value shows the mean (±S.D.) of B/B_0 (n =5). The linearity was good from 0.1 to 3 ng mL^{-1} which has a linear regression equation and acceptable correlation (R^2).



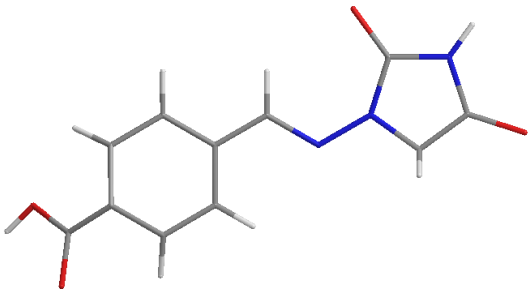
Nitrofurantoin



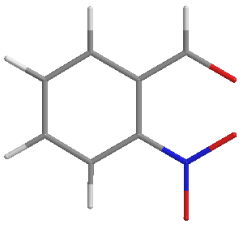
AHD



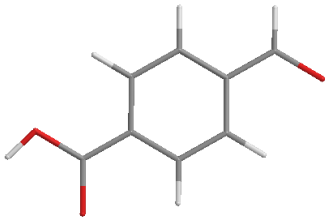
2-NP-AHD



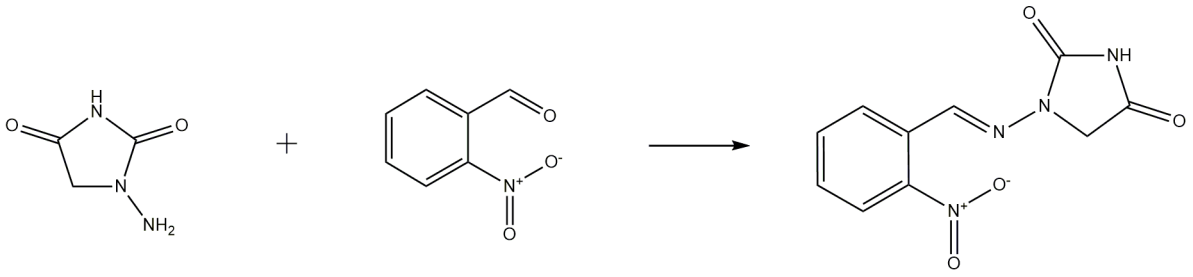
4-CP-AHD



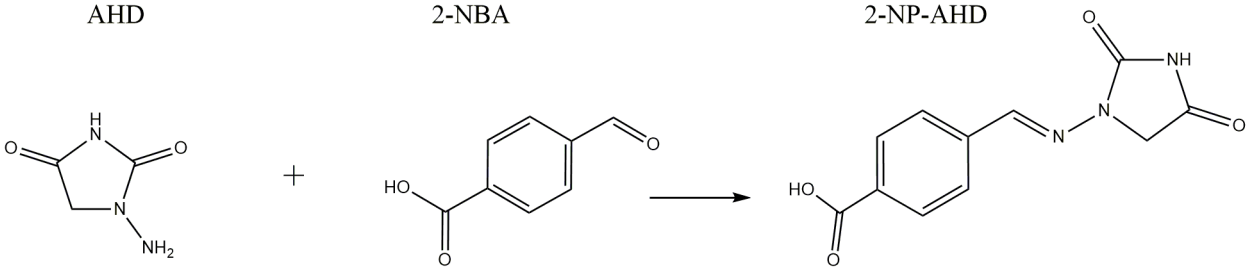
2-NBA



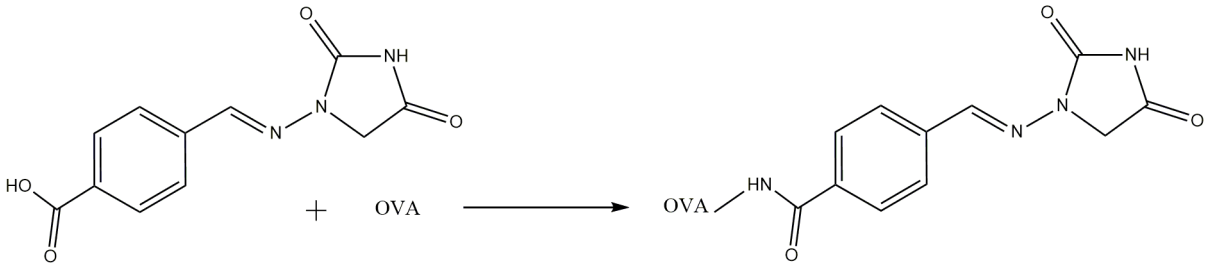
4-CBA



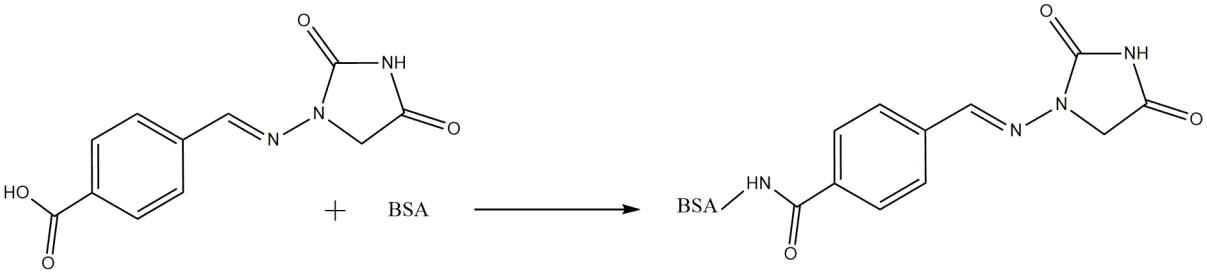
AHD 2-NBA 2-NP-AHD



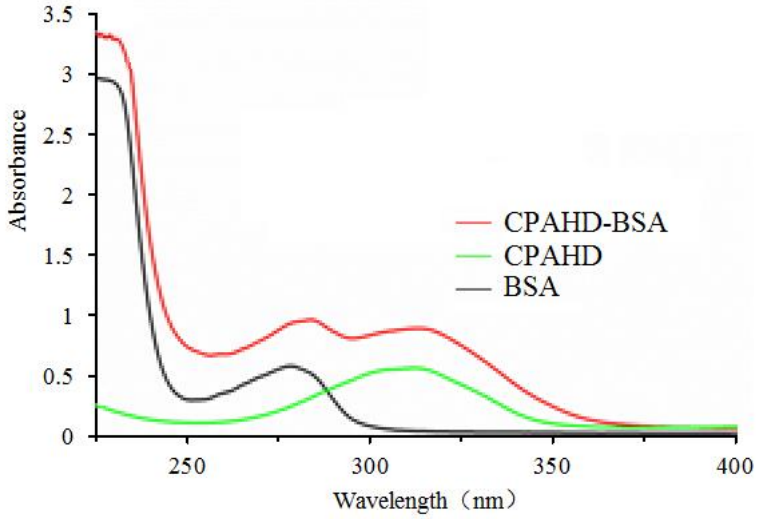
AHD 4-CBA 4-CP-AHD

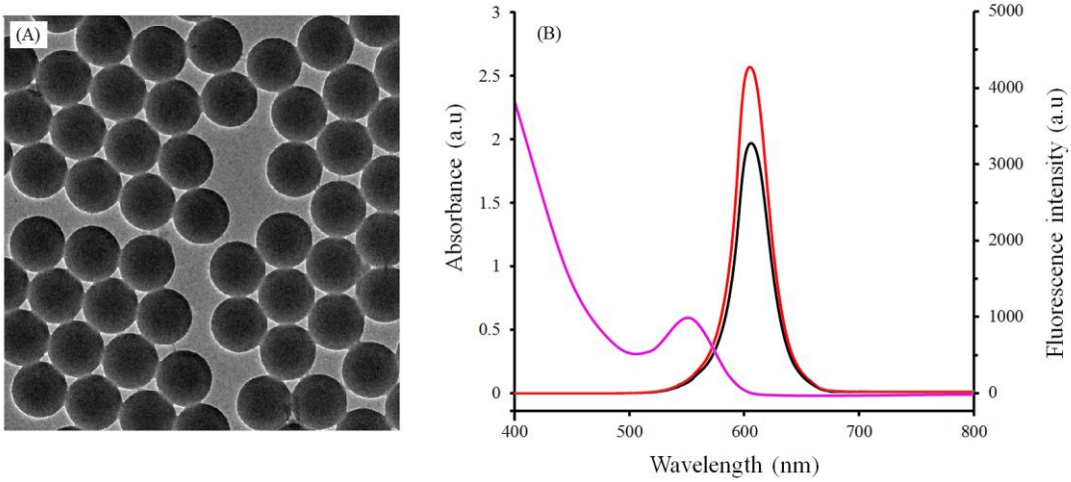


4-CP-AHD 4-CP-AHD-OVA



4-CP-AHD 2-CP-AHD-BSA





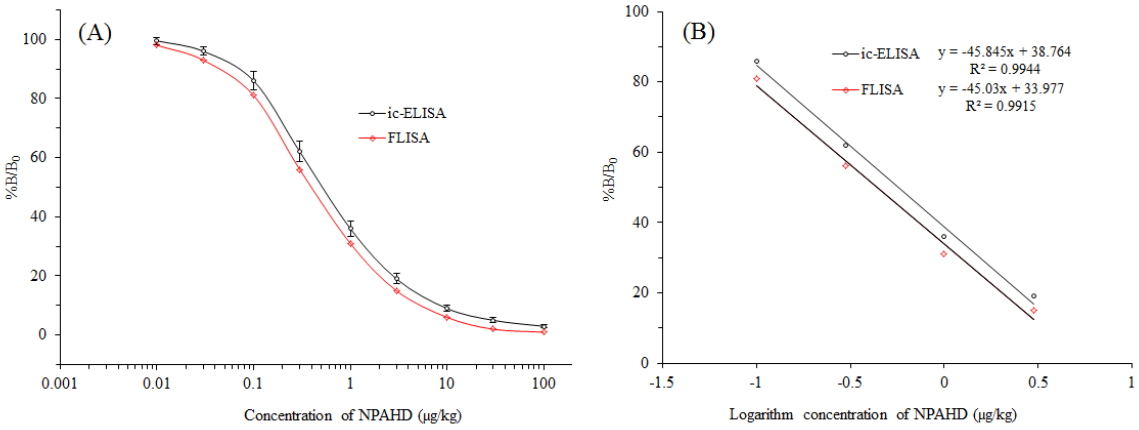


Table 1.

IC_{50} values and cross-reactivities of selected compounds tested by ic-ELISA in this study and in the literature.[a]

Compound	Monoclonal antibody 3.2.4/5A8		Monoclonal antibody [21]		Monoclonal antibody [19]		Monoclonal antibody [20]		Polyclonal antibody [4]	
	IC_{50}[b]	%CR	IC_{50}	%CR	IC_{50}	%CR	IC_{50}	%CR	IC_{50}	%CR
NPAHD	0.57	100	0.68	100	5.31	100	0.60	100	15	100
Nitrofurantoin	>1000	<0.1	>1000	<0.1	0.6	876	–	<0.01	3.2	468.8
AHD	38.24	14.91	92	0.74	–	<0.3	–	<0.01	260	5.8
CPAHD	0.77	74.03	ND[c]	–	1.91	287	1.51	39.67	0.2	7500
NPAOZ	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
Furazolidone	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
AOZ	>1000	<0.1	>1000	<0.1	ND	–	–	<0.01	ND	<0.1
CPAOZ	>1000	<0.1	ND	–	ND	–	–	<0.01	ND	–
NPAMOZ	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
furaltadone	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
AMOZ	>1000	<0.1	>1000	<0.1	ND	–	–	<0.01	>5000	<0.1
CPAMOZ	>1000	<0.1	ND	–	ND	–	–	<0.01	ND	–
NPSEM	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
nitrofurazone	>1000	<0.1	>1000	<0.1	–	<0.3	–	<0.01	>5000	<0.1
SEM	>1000	<0.1	>1000	<0.1	ND	–	–	<0.01	ND	<0.1
CPSEM	>1000	<0.1	ND	–	ND	–	–	<0.01	ND	–

2-nitrobenzaldehyde	61.71	0.92	57	1.19	ND	–	–	<0.01	>5000	<0.1
4-carboxybenzaldehyde	47.22	1.21	35	1.95	ND	–	–	<0.01	>5000	<0.1

[a] All data was calculated using the percentage of cross-reactivity (CR) of NPAHD as 100%.

[b] The units of IC_{50} are ng mL^{-1}, IC_{50} was the competitor concentration at which the absorbance value was decreased by half compared to the absorbance value of no competitor.

[c] ND= Not detected.

Table 2.

Mean recoveries and coefficients of variation for the AHD in edible animal tissues using optimized ELISA and FLISA (n=5) .

Sample	AHD content fortified in sample (μg kg^{-1})	Theoretical AHD concentration in extract (ng mL^{-1})	ELISA				FLISA			
			Intra-assay		Inter-assay		Intra-assay		Inter-assay	
			Recovery ± SD (%)	CV (%)	Recovery ± SD (%)	CV (%)	Recovery ± SD (%)	CV (%)	Recovery ± SD (%)	CV (%)
Catfish	0.06	0.2	83.7 ± 8.9	10.6	85.7 ± 9.6	11.2	82.5 ± 7.6	9.2	84.6 ± 8.8	10.4
	0.15	0.5	89.8 ± 6.5	7.2	84.9 ± 7.4	8.7	82.9 ± 8.4	10.1	82.4 ± 7.5	9.1
	0.6	2	81.5 ± 8.7	10.7	83.6 ± 9.2	11.0	95.8 ± 8.5	8.9	102.3 ± 8.6	8.4
	1.5	5	98.8 ± 9.6	9.7	103.2 ± 9.4	9.1	88.3 ± 7.3	8.3	94.7 ± 9.7	10.2
Crab	0.06	0.2	103.7 ± 8.4	8.1	113.7 ± 9.1	8.0	89.4 ± 9.5	10.6	92.2 ± 8.5	9.2
	0.15	0.5	86.4 ± 9.4	10.9	103.8 ± 8.9	8.6	90.2 ± 8.8	9.8	97.4 ± 6.3	6.5
	0.6	2	87.3 ± 9.3	10.7	96.3 ± 10.3	10.7	107.6 ± 9.4	8.7	100.7 ± 7.6	7.5
	1.5	5	112.8 ± 9.6	8.5	95.2 ± 7.6	8.0	89.2 ± 6.4	7.2	94.3 ± 7.6	8.1
Shrimp	0.06	0.2	89.7 ± 8.8	9.8	93.1 ± 9.5	10.2	103.7 ± 8.6	8.3	94.8 ± 8.8	9.3
	0.15	0.5	85.7 ± 10.4	12.1	90.7 ± 7.9	8.7	86.4 ± 7.5	8.7	98.7 ± 10.3	10.4
	0.6	2	88.6 ± 8.3	9.4	89.5 ± 9.4	10.5	91.7 ± 8.2	8.9	96.1 ± 8.2	8.5
	1.5	5	80.3 ± 9.5	11.8	85.2 ± 9.7	11.4	82.9 ± 7.8	9.4	89.3 ± 7.5	8.4

SD:standard deviation; CVs:coefficients of variation

Table 3.

Comparison of results obtained using ic-ELISA, FLISA and reference LC-MS/MS method.

Type of sample	Number of sample	Results (μg kg^{-1})		
		ic-ELISA	FLISH	LC-MS/MS
Carp	10	n.d.[a]	n.d	n.d
Grass carp	6	n.d	n.d	n.d
Crucian carp	9	n.d	n.d	n.d
Chub	5	n.d	n.d	n.d
Catfish	5	n.d	n.d	n.d
Shrimp	6	n.d	n.d	n.d
Chicken muscle	8	n.d	n.d	n.d
Swine muscle	7	n.d	n.d	n.d
Milk	9	n.d	n.d	n.d
Cattle muscle	5	n.d	n.d	n.d

Note: [a] n.d., Not detected.