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Hapten synthesis, monoclonal antibody production and immunoassay development for direct detection of 4-hydroxybenzehydrazide in chicken, the metabolite of nifuroxazide

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ABSTRACT

Derivatization is usually employed in immunoassay for detection of metabolites of nitrofurans and avoiding derivatization could be preferable to achieve an efficient screening. In the study, we designed four haptens of 4-hydroxybenhydrazide (HBH), the nifuroxazide metabolite. The effect of hapten structures on antibody affinity were evaluated and one monoclonal antibody was produced by using the Hapten C with a linear alkalane spacer arm. After optimization, an enzyme linked-immunosorbent assay (ELISA) was established with an 50% inhibition concentration of 0.25 ng mL^{-1} for HBH, which could ensure the direct detection of HBH without derivatization. The limit of detection of the ELISA for HBH was 0.12 μg kg^{-1} with the recoveries of 90.1–96.2% and coefficient of variation (CV) values lower than 9.1%. In conclusion, we produced several high affinity antibodies to HBH with new designed hapten and developed an icELISA for the direct detection of HBH without derivatization in chicken.

1. Introduction

Nitrofurans are a class of synthetic broad-spectrum antibacterial agents which are characterized by a distinctive 5-nitrofuran group that were previously used to treat the gastrointestinal and dermatological complications in the poultry, bee, aquaculture and as well as used as growth promoters (Mottier et al., 2005; Radovnikovic et al., 2011). The use of nitrofurans drugs in food animal production has been banned due to the carcinogenic and mutagenic effects on human health by European Union (Commission Regulation No 1442/95). The European Union declared the minimum required performance limits (MRPLs) for nitrofuran are set to be 1.0 μg kg^{-1} in poultry meat and aquaculture products by Commission Decision, 2003/181/EC. However, nitrofurans are still illegally used in veterinary practice due to their low cost, and effectiveness, particularly in developing countries (Xu et al., 2013). From 2002 to 2015 there were 826 rapid alerts issued for nitrofurans in a wide range of products from various countries (Cooper et al., 2017). Nitrofurans are metabolized quickly *in vivo* and do not persist in edible tissues. However, the metabolites of these drugs bind to tissue proteins and persist for considerable periods in animal tissues after treatment. Since, the metabolites of nitrofurans are too small to detect directly, derivatization with 2-nitrobenzaldehyde (2-NBA) to form nitrophenyl (NP) derivatives before detection was usually used (Cooper et al., 2005). The drawbacks of derivatization procedure include the wastage of time and labor, usually requiring up to 16 h to complete (Jester et al., 2014; Liu et al., 2017; Pimpitak et al., 2009; Xu et al., 2013). Apparently, avoiding derivatization can be preferable to achieve an efficient screening by shortening the analysis time, reducing the cost and labor in an indirect competitive enzyme linked-immunosorbent assay (icELISA), if high affinity antibody is available. Nowadays, a few reports have been published focusing on direct detection of nitrofuran metabolites in food samples, including 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ) (Liu et al., 2017; Song et al., 2012) and 3,5-dinitro-salicylic acid hydrazide (DNSH) (Shen et al., 2008).

Nifuroxazide (NFX) is an important member of nitrofurans patented since 1966 and possibly used as a growth-promoter and for the

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treatment in poultry production. NFX was undetectable in some chicken tissues, i.e., liver, kidney, muscle and plasma after 14 days of cessation, while, the metabolite, 4-hydroxybenzhydrazide (HBH), was detectable even after 28 days of cessation (Feng et al., 2013). Therefore, considering the consequences on human health and international trade, it is necessary to establish an appropriate, rapid, cost effective and accurate analytical technique to detect the HBH in animal tissues. Until now, only a few instrument-based analytical methods have been developed to detect NFX and HBH in animal feed by high performance liquid chromatography (Feng et al., 2013) and liquid chromatography tandem mass spectrometry (Barbosa et al., 2007). Recently, a class-specific polyclonal antibody based on icELISA was reported to screen seven parent nitrofurans drugs, including NFX, furazolidone (FZD), nitrofurantoin (NFT), nitrofurazone (NFZ), furaltadone (FTD), nifursol (NFS) and nifurstyrenate (NFSS) in animal feed with low sensitivity and could not recognize their metabolites (Li et al., 2010).

Antibody is the key reagent which governs the sensitivity and specificity of an icELISA. To produce a desired antibody to HBH, the structure of the hapten has been regarded as the most important factor, requiring maximum the steric, hydrophobic, and electronic similarities of a hapten to the target of interest. Since frequently used derivative reagents to prepare haptens of nitrofuran metabolite are not appropriate to obtain a high affinity antibody. In the present study, we designed and synthesized haptens of HBH by using new derivative reagent as spacer arms to achieve the purpose of direct detection of HBH without derivatization. After antigen preparation and immunization to mice, we obtained high affinity monoclonal antibodies (mAbs) and the icELISA was developed for the direct detection of HBH metabolite in chicken tissues.

2. Materials and methods

2.1. Materials

NFX was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). HBH was acquired from Amethyst chemicals (Beijing, China). NBA was purchased from Aladdin (Shanghai, China). Nitrovin was supplied by J & K Scientific (Beijing, China). 4-Formylphenoxy acetic acid (4-FPA), ethyl 6-bromohexanoate, 5-hydroxy-2-nitrobenzaldehyde, ethyl 2-bromoacetate, p-hydroxyl-benzoyl hydrazine and oxamic-hydrazide were obtained from TCI (Shanghai, China). 3,5-dinitro-salicylic acid hydrazide and aminogunidine hydrochloride, were purchased from Fluorochem (Hadfield, UK). Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), hypoxanthine aminopterin thymidine (HAT), complete and incomplete Freunds adjuvant, polyethylene glycol (PEG) 1500, and fetal calf serum were procured from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP) labeled goat anti-mouse immunoglobin G (IgG) was purchased from Jackson Immuno Reaserch Laboratories (West Grove, PA, USA). Dulbecco's modified eagle medium (DMEM) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 3,3,5,5-Tetramethylbenzidine (TMB) substrate, *N, N*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from Aladdin Reagent (Shanghai, China). Sodium chloride (NaCl), dimethylformamide (DMF) and sodium hydroxide (NaOH) were supplied by Sinopharm Chemical Reagent (Beijing, China). 96-well microtiter plates and cell culture plates were obtained from Corning Life Sciences (New York, USA). Female BALB/c mice were supplied by Vital River Laboratory Animal Technology (Beijing China). Chicken samples were provided by the National Reference Laboratory for Veterinary Drug Residues (Beijing, China). Buffer solutions, including coating buffer, antibody dilution buffer, blocking buffer, substrate solution, washing buffer and stop buffer used in this study were listed in *Supplementary material*.

2.2. Synthesis and characterization of haptens and derivatives

The synthetic routes of all haptens and nitrophenyl-4-hydroxybenzhydrazide (NPHBH) are shown in Scheme 1. The identified data of haptens and NPHBH by liquid chromatography triple quadruple mass spectrometry (Agilent Technologies, Santa Clara, USA) and nuclear magnetic resonance (NMR) spectrometry DRX-400 (Bruker, Rheinstetten, Germany) are provided in *Supplementary material* (Fig. S1 and Fig. S2).

2.2.1. Hapten A. 2-(4-((2-(4-hydroxybenzoyl)hydrazono)methyl) phenoxy)acetic acid (2)

Briefly, 1.0 mmol HBH in 5 mL methanol was added to a stirring solution of 1.5 mmol 4-FPA in 10 mL methanol for 3 h at room temperature followed by washing with ethanol four times to eliminate unreacted reagents. After removing methanol by vaporization, Hapten A was obtained (yield, 80%). HRMS (*m/z*) calc. for $C_{16}H_{14}N_2O_5$, 314.30, found 313.00 $[M-H]^-$. ^1H NMR: δH (400 MHz, DMSO–d6): 7.4840 (m, 1H, ArH), 8.3435 (m, 1H, ArH), 8.4453 (m, 1H, ArH), 8.5970–8.5858 (m, 1H, $J = 3.36$ Hz, ArH), 8.8397–8.8265 (m, 1H, $J = 3.96$ Hz, ArH), 13.8882 (s, 1H, COOH).

2.2.2. Hapten B. (6-(2-(4-hydroxybenzoyl) hydrazineyl) hexanoic acid) (3)

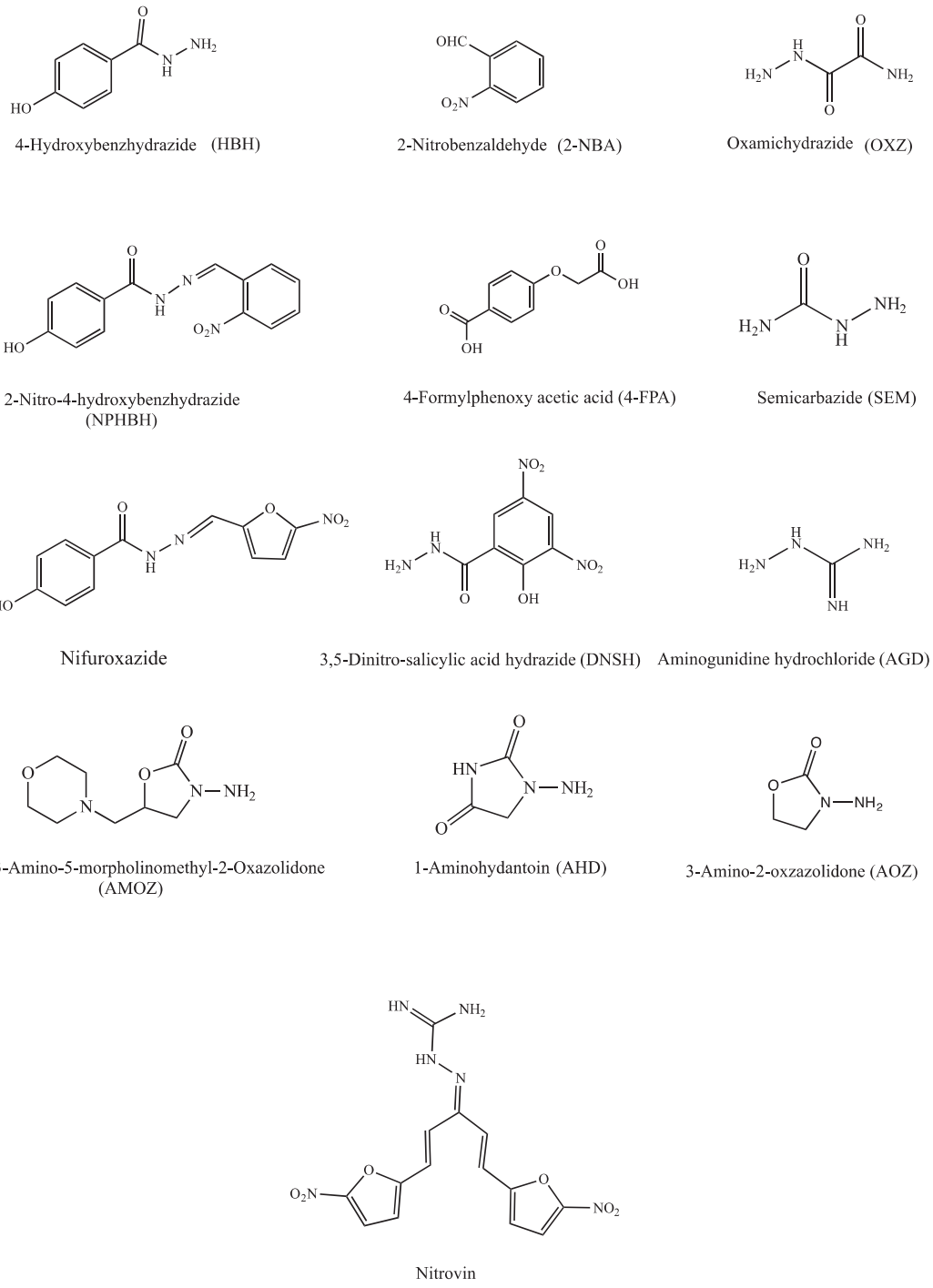
Briefly, 1.0 mmol HBH and 1.0 mmol ethyl 6-bromohexanoate dissolved in DMF, and added to 2 M sodium acetate as a reactant solution and left on stirring at 35 °C for 24 h. The residues were obtained by vacuum drier after washing with water. The precipitates were recrystallized in petroleum ether and ethyl acetate (1:1, v/v) and a yellow solid powder was obtained (yield 50%). The powder was dissolved in methanol and added dropwise to 1 mol L^{-1} sodium hydrate and left on stirring at 30 °C for 2 h. After reaction, the mixture was poured into water and pH value was adjusted to 4 by adding acetic acid and then left on ice water bath stirring for 1 h. The precipitates were dried and Hapten B was obtained (yield, 90%). HRMS (*m/z*) calc. for $C_{13}H_{18}N_2O_4$, 266.30, found 267.13 $[M-H]^+$. ^1H NMR (400 MHz, DMSO–d6): δ 9.91 (s, 1H, NH), 9.7 (s, 1H, OH), 7.70 (d, $J = 8.5$ Hz, 2H, ArH), 6.80 (d, $J = 8.5$ Hz, 2H, ArH), 3.38 (t, $J = 7.0$ Hz, 2H, CH_2), 2.77 (t, $J = 7.3$ Hz, 2H, CH_2), 1.91 (m, 4H, CH_2), 1.56–1.29 (m, 2H, CH_2).

2.2.3. Hapten C.(2-(3-((2-(4-hydroxybenzoyl) hydrazono) methyl)-4-nitrophenoxy) acetic acid) (5)

Briefly, 1.0 mmol 5-hydroxy-2-nitrobenzaldehyde, 1.0 mmol ethyl 2-bromoacetate and 2.0 mmol potassium carbonate were dissolved in acetonitrile and refluxed for 3 h. Then the mixture was evaporated to remove the acetonitrile and the obtained precipitate was re-dissolved in water. The mixture was extracted three times with ethyl acetate, then washed with water and evaporated to collect the product (yield 80%). The obtained product was dissolved in methanol and 3.0 mmol sodium hydrate solutions was added for reaction at 30 °C for 1 h. The pH value of the mixture was adjusted to strong acidic state by adding 6 M hydrochloric acid to obtain the precipitate and dried under vacuum to get yellow powder (yield, 60%). Then, 1.0 mmol product and 1.0 mmol HBH were dissolved in ethanol and refluxed for 5 h. The reaction mixture was cooled to room temperature, then filtered and dried to obtain the light-yellow powder (yield, 90%). HRMS (*m/z*) calc. for $C_{16}H_{13}N_3O_7$, 359.29, found 360.08 $[M-H]^+$. ^1H NMR (400 MHz, DMSO-d_6): δ 12.3 (s, 1H,COOH), 8.10 (s, 1H, NH), 8.14 (s, 1H,OH), 7.95 (s, 1H, CHO), 7.49 (d, $J = 9.1$ Hz, 1H, ArH), 7.21 (d, $J = 8.3$ Hz, 2H, ArH), 6.89 (s, 1H, ArH), 7.16 (dd, $J = 9.1$, 2.3 Hz, 1H, ArH), 4.91 (d, $J = 8.4$ Hz, 2H, ArH), 3.38 (s, 2H, CH_2), 2.51 (p, $J = 6.7$ Hz, 2H, CH_2).

2.2.4. Hapten D. 4-(3-((2-(4-hydroxybenoyl)hydrazono)methyl)-4-nitrophenoxy) butanoic acid) (6)

The procedure of Hapten D was similar to that of Hapten C as shown in Scheme 1. Briefly, in the process, 1.0 mmol ethyl 4-bromobutanoate was used in the synthesis of Hapten D instead of ethyl 2-bromoacetate in the synthesis of Hapten C. HRMS (*m/z*) calc. for $C_{18}H_{17}N_3O_7$, 387.35, found 388.11 $[M-H]^+$, ^1H NMR (400 MHz, DMSO-–d6): δ 12.04



Scheme 1. The synthesis routes of HBH haptens.

(m, 2H, NH, COOH), 8.95 (s, 1H, OH), 8.15 (s, 1H, CHO), 7.85 (d, J = 9.1 Hz, 1H, ArH), 7.50 (d, J = 8.5 Hz, 2H, ArH), 7.21 (s, 1H, ArH), 7.17 (d, J = 9.1, 2.5 Hz, 1H, ArH), 6.89 (d, J = 8.5 Hz, 2H, ArH), 4.19 (t, J = 6.3 Hz, 2H,CH_2), 3.37 (t, J = 7.2 Hz, 2H, CH2), 2.52 (p, J = 6.7 Hz, 2H, CH_2) 1.06 (p, J = 6.2 Hz, 2H, CH_2).

2.2.5. *4-hydroxy-N'-(2-nitrobenzylidene)benzohydrazide (NPHBH).*

Briefly, 1.0 mmol *o*-nitrophenyl formaldehyde and 1.0 mmol *p*-hydroxyl-benzoyl hydrazine were dissolved in 5 mL anhydrous ethanol and refluxed for 3 h then cooled at a room temperature. The residues were then dried and a pale white powdery solid was obtained (yield, 90%). HRMS (m/z) calc. for $C_{14}H_{11}N_3O_4$, 285.26, found 286.08 $[M-H]^+$, ^1H NMR (400 MHz, DMSO-–d6): δ 11.97 (s, 1H, NH), 10.15 (s, 1H, OH), 8.81 (s, 1H, CHO), 8.09 (d, J = 7.0 Hz, 1H, ArH), 8.04 (d, J = 8.2 Hz, 1H, ArH), 7.81 (d, J = 8.2 Hz, 2H, ArH), 7.77 (d, J = 7.8 Hz, 1H, ArH), 7.64 (t, J = 7.8 Hz, 1H, ArH), 6.84 (d, J = 8.6 Hz, 2H, ArH).

2.3. *Preparation of immunogens and coating antigens*

The four haptens with carboxylic acid group was activated and coupled to KLH and BSA through the active-ester method (Wang et al., 2013). Firstly, each hapten (0.1 mmol) was dissolved in mixture of NHS (0.15 mmol) and DCC (0.1 mmol) in 0.5 mL DMF. Then the reacted mixture was kept on stirrer at 4 °C for 6 h. After the removal of

precipitates, the supernatant of each hapten solution was divided into two parts and then dropwise added into 9.5 mL phosphate buffered saline (PBS) solution containing 20 mg KLH and 9.5 mL PBS containing 20 mg BSA, respectively. The solutions containing the hapten-protein conjugates were stirred for overnight and then conjugates were dialyzed in PBS for three days at 4 °C. The Haptens-KLH were stored at −70 °C and Haptens-BSA were stored at −20 °C until used. The BSA conjugates were then characterized by the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF-MS) (Bruker, Rheinstetten, Germany) and the hapten-ratios were calculated by following formula and provided in *Supplementary material*:

$$\text{Molar ratio} = \{\text{MW (conjugates)} - \text{MW (BSA)}\}/\text{MW (haptens)} \quad (1)$$

2.4. Generation of monoclonal antibody

All animal treatments were in strict accordance with the Chinese laws and guidelines and were approved by the Animal Ethics Committee of China Agricultural University. Twenty female BALB/c mice (8–10 weeks old) were randomly divided in to four groups and subcutaneously immunized with immunogens three times with an interval of three weeks. The first dose was given through injection of 100 μg of immunogen in 0.25 mL of 0.9% NaCl and 0.25 mL of Freund's complete adjuvant. Three and six weeks after the initial injection, mice were boosted with 100 μg of immunogen in Freund's incomplete adjuvant (Wang et al., 2018). Ten days after the third immunization, the sera (approximately 50 μL) were collected and assayed to analyze the sensitivity and specificity by the icELISA. Values of optical density (OD) of the icELISA were measured by using PerkinElmer Envision plate reader (Waltham MA, USA). The mice with a low 50% inhibition concentration (IC_{50}) of HBH were selected for subsequent cell fusion. The cell fusion was carried out as our previous report (Wang et al., 2015). The selected mice were injected intraperitoneal with 300 μg immunogen. After three days, the spleen cells of boosted mice were hygienically harvested and fused with SP2/0 myeloma cells in a 10:1 ratio by adding PEG 1500 as the fusing mediator (Wang et al., 2015). The fusion cells were cultured in HAT and positive hybridomas were screened after eight days of fusion. Sub-cloning was carried out three times by limiting dilution method and optimum single cell mass possessing high titer and high affinity to HBH was collected and cultured. One part of the hybridoma was cryopreserved and other parts were collected to produce ascites via intraperitoneal injection in adult female BALB/c mice (Zhang et al., 2016).

2.5. Development and optimization of icELISA

The dilution of coating antigens and mAbs were optimized by the checkerboard (ArunKumar et al., 2012). In brief, the microtiter plate coated with antigens (100 μL $well^{-1}$) was diluted in coating buffer and then incubated at 37 °C for 2 h. Followed by washing step blocked with 150 μL $well^{-1}$ blocking buffer and placed in incubator at 37 °C for 1 h, buffer solution in the plates were discarded. Then 50 μL $well^{-1}$ of standard solution of HBH (or other competitors) was added and 50 μL $well^{-1}$ of diluted antibody solution were added into microtiter plate, respectively. After incubation of 30 min at 37 °C, the plates were washed with washing buffer for three times. Goat anti-mouse IgG (HRP labeled) (1:5000, 100 μL $well^{-1}$) was then added and incubated for 30 min at 37 °C. After rinsing three times, 100 μL $well^{-1}$ of the mixed TMB substrate solution was added in each well and incubated for 15 min at 37 °C. Finally, the reaction was halted by addition of 2 mol L^{-1} H_2SO_4 (50 μL $well^{-1}$) and the OD values of 450 nm were measured immediately. The standard curve was generated as described previously using the following equation: (Suryoprabowo et al., 2014).

$$Y = (A - B)/[1 + (X/C)D] + B \quad (2)$$

where A and B are the response at high and low asymptotes of the curve, respectively. C is the concentration of the targets in 50% inhibition. D is the slope at the inflation point of the sigmoid and X is the calibration concentration.

The cross reactivities (CRs) of the icELISA were calculated by using subsequent formula:

$$CR = IC_{50} \text{ of HBH}/(IC_{50} \text{ of competitor compounds}) \times 100\% \quad (3)$$

To enhance the performance of the ELISA, the concentrations and pairing combinations of coating antigen and mAb, and other physiological parameters such as temperature, pH values, ionic strength, ethyl acetate and hexane concentrations were analyzed. The effects of these parameters were evaluated by the maximum absorbance (A_{max}, the absorbance value at zero analyte concentration) and the highest ratio of A_{max} /IC_{50}.

2.6. Conformational studies and electronic Analysis.

All structures were built in Gaussian 09 (Gaussian Inc., Wallingford, CT, USA) according to the configurations in the PubChem database. The structure ligands were minimized based on CHARMm force field, and then the molecules were aligned using HBH as the template molecule in Discovery Studio 2018 (Accelrys Software, Inc., San Diego, CA, USA). Density functional theory calculated with the B3LYP functional and 6–311^{++}G (d, p) basis set were performed to calculate atom charges and electron density surface features using the Gaussian 09 package. The Gaussian 09 and Gaussian View 5 packages were used to display the molecular electrostatic potential (ESP).

2.7. Sample pretreatment and recovery test

The chicken samples were provided by the National Reference Laboratory for Veterinary Drug Residues (Beijing, China). To examine the accuracy and precision of the developed icELISA, recovery studies were performed with total 20 spiked chicken samples. The sample treatment was conducted according to a previous report (Song et al., 2012). Briefly, 1.0 g of homogenized chicken was spiked with HBH standards at the final concentrations of 0.5, 1.0, 1.5 and 2.0 μg kg^{-1} respectively. Then, 4.0 mL of deionized water and 0.5 mL of 1 mM hydrochloric acid were added and subjected to ultrasound for 20 min and incubated for 30 min in water bath at 60 °C to release the bound HBH from the tissue. Then, 5.0 mL of 0.1 mM dipotassium phosphate was added and pH value was then adjusted to 7.0 with addition of 0.4 mL of 1 mM sodium hydrate. After adding 6.0 mL of ethyl acetate, mixture was vortexed for 10 min and centrifuged at 5000 rpm for 10 min. Finally, 3 mL of supernatant was collected and dried under nitrogen at 60 °C, then residues were re-dissolved in 1.0 mL of *n*-hexane containing 1.0 mL of 0.02 M PBS (pH 7.4) and centrifuged at 5000 rpm for 5 min. After removing *n*-hexane, 50 μL of extract was separated for subsequent use in the icELISA.

3. Results and discussion

3.1. Hapten design, synthesis and preparation of conjugates

The aim of this study was to develop icELISA for the direct detection of HBH without derivatization step after production of a high affinity antibody. In the process of antibody preparation for small molecules, hapten design play an important role in governing the affinity and specificity of the obtained antibody. In previous reports for the hapten design of nitrofurans, metabolites such as 3-Amino-2-oxazolidinone (AOZ), AMOZ, 1-aminohydantoin (AHD) and semicarbazide (SEM) as shown in Figs. 1, 3 -carboxybenzaldehyde (3-CBA) and 4-FPA were the most frequently used derivatizing agents for preparation of haptens (Chadseesuwan et al., 2013; Cooper et al., 2004; Pimpitak et al., 2009; Song et al., 2012; Zhang et al., 2010). However, the resultant antibody obtained by CBA and FPA modified metabolites usually showed high affinity to NP and carboxyphenyl (CP) derivatives such as NPAOZ,

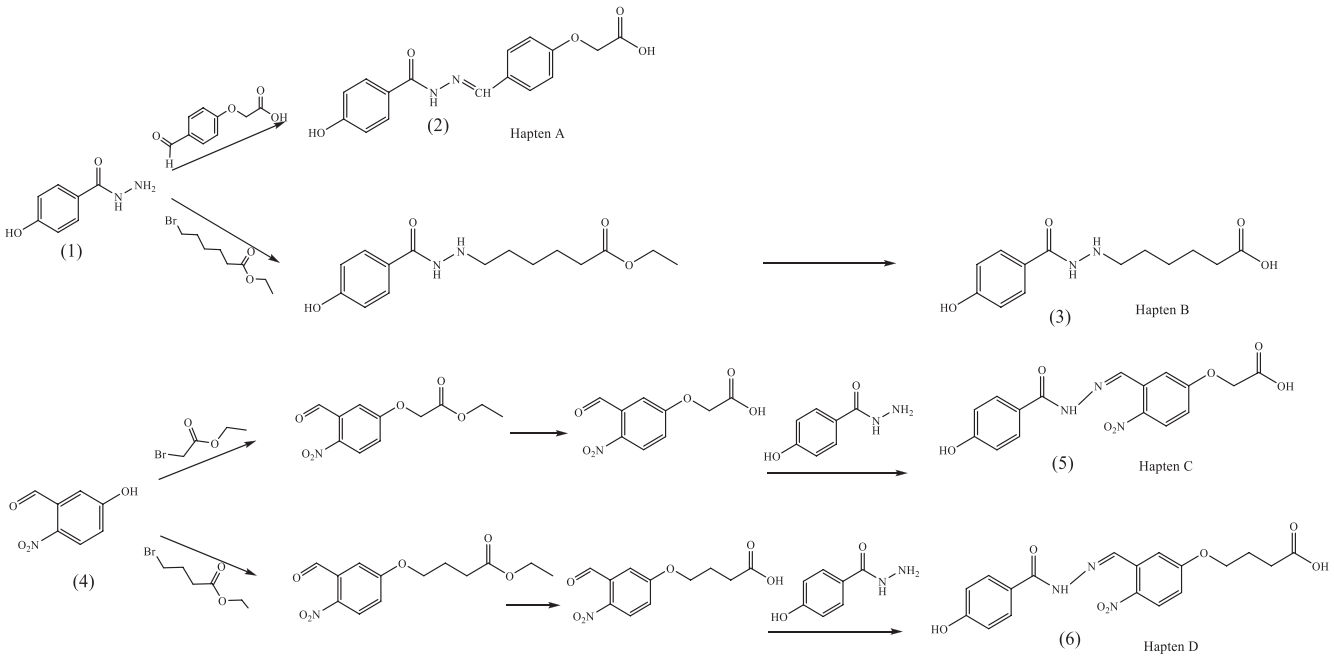


Fig. 1. Chemical structures of NFX, HBH, other nitrofurans and metabolites.

CPAOZ and low affinity to their metabolites (Xu et al., 2013). Other derivative reagents such as maleic anhydride, nitrophenyl-hexanoic acid and glyoxylic acid were also employed to modify metabolites to prepare haptens and few good antibodies were produced by these alternatives (Cheng et al., 2009; Liu et al., 2015; Shen et al., 2008, 2007). In addition, some authors attempted to develop haptens by directly introducing long linear aliphatic chains between metabolite and protein, resulting in poor recognition ability to both parent drugs and metabolites (Cooper et al., 2004). Until now, there are only a few reports that have focused on production of antibodies that can recognize free metabolites from food samples without derivatization step, i.e., AMOZ, with an IC_{50} values of 4.1 ng mL^{-1}, 1.15 ng mL^{-1} and DNSH with an IC_{50} values of 0.217 nmol mL^{-1}, However, these were not enough to use in development of highly sensitive icELISA (Liu et al., 2017; Song et al., 2012; Shen et al., 2008). The reason for using derivative reagents to prepare hapten and then detection of derivatized metabolite is attributed to small size of metabolites and more steric hindrance of the metabolite epitope when coupling to carrier protein and then reduced immunogenicity. In order to avoid derivatization step during sample pretreatment, the antibody should provide sufficient affinity to HBH. Thus, in the present study a new hapten design strategy was planned to obtain highly sensitive mAb and to develop icELISA-based detection method.

Generally, an appropriate hapten should mimic the target molecule as much as possible in terms of structural resemblance, size, steric conformation, electronic configuration and hydrophobic properties (Maximilian & Anja, 2014). In case of a very small molecule like HBH (mw152.15), a rationally derivative reagents used as spacer arm must be introduced to support the certain epitope away from carrier protein and maximally expose it to immune system of animal. As shown in Scheme 1, we have designed four HBH haptens with different spacer compositions and lengths. Hapten A was synthesized by using a previously reported 4-FPA as spacer, while, Hapten B was synthesized by introducing a long linear aliphatic chain of six atoms by using ethyl 6-bromohexanoate. Hapten C and Hapten D were synthesized in three steps and possessed similar structures only differing in the length of spacer. A key point in the design of Hapten C and Hapten D was the use of 5-hydroxy-2-nitrobenzaldehyde as a derivative reagent and then lengthen by ethyl 2-bromoacetate and ethyl 4-bromobutanoate, respectively. The structures of HBH hapten we designed in the present study are expected to provide a higher possibility to induce high specificity and affinity antibody response to NPHBH and possibly HBH alone due to less steric hindrance from carrier protein and more resemble mimic. The rationality of the designed haptens was firstly studied by conformational studies and electronic analysis. We aligned HBH, Hapten A, B, C and D based on their lowest conformations, setting HBH as the template molecule. As shown in Fig. 2a, the HBH moiety of haptens and target compound HBH were overlapped perfectly, which illustrating that the introduction of spacer arms at N11 position (insert section of Fig. 2b) of HBH can hardly influence the conformational feature of HBH. In addition, the introduction of different spacer arms didn't cause the significant alteration of atomic charges in HBH moiety (Fig. 2b), e.g., atomic charges difference of HBH and four haptens were less than 0.1 a.u at C1-C8 and O3 position. Furthermore, the atomic charges distribution of O9/N10/N11 position varied differently, that can be affected by the tethered spacer arms. As depicted above, the four haptens mimicked the target HBH well in conformational and electronic features, which demonstrating that the four haptens in this study can be used to prepare antibodies for HBH. In addition, among the four haptens (Hapten A, B, C and D), Hapten C shared the most similar atomic charges distribution to HBH, rendering it to be a promising hapten. For the HBH hapten design, it is envisaged that a linear aliphatic spacer arm may not be enough to elicit a significant antibody response due to the infinitesimal and simple structure. The introduction of appropriately complicated and bulked structure is expected such as phenyl group, that will act not only a spacer arm but also assist in triggering the immune response by increasing the size of resultant hapten.

All haptens and derivative were identified by mass spectrum and nuclear magnetic resonance, providing in *Supplementary material* (Figs. S1 and S2). In addition, the nitrobenzaldehyde carboxylic acids of haptens were activated by NHS and DCC, then conjugated with KLH and BSA. An observable shift in the maximum peak of the BSA conjugates in comparison to the control was observed in the Fig. S3, indicating that haptens were successfully conjugated to the carrier protein. The calculated molar ratios of the hapten-BSA were 11.4:1 (Hapten A-BSA), 14.4:1 (Hapten B-BSA), 8.2:1 (Hapten C- BSA) and 10.4:1 (Hapten D-BSA), respectively.

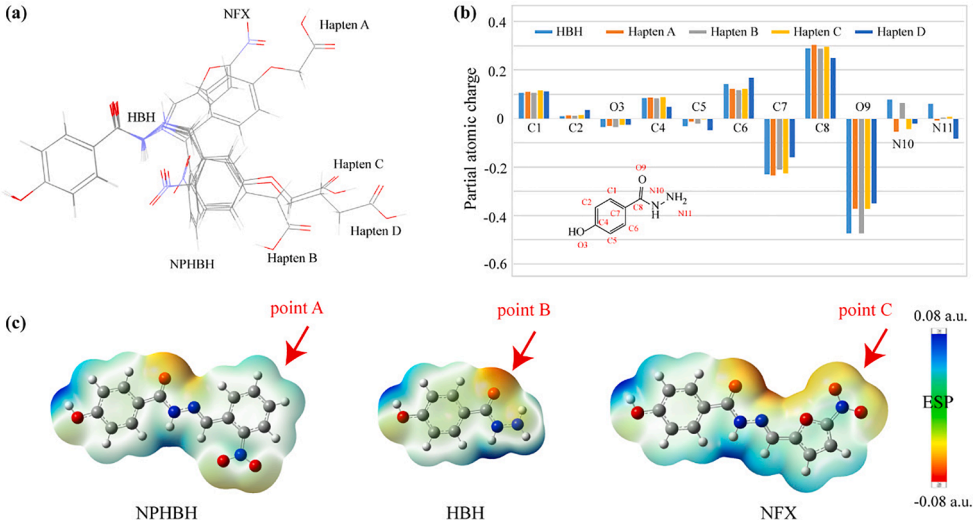


Fig. 2. Conformational and electronic features of HBH, NPHBH, NFX and haptens, (a) overlap of lowest energy conformers of HBH, NPHBH, NFX, Hapten A, B, C and D, (b) calculated partial atomic charges of HBH, Hapten A, B, C and D, (c) the ESP of NPHBH, HBH and NFX. The negative ESP regions are indicated in red, and the positive regions in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Antiserum characterization and monoclonal antibodies production

Each hapten–KLH conjugate was used to immunize a set of mice. The effectiveness of haptens on antibody response was evaluated by antibody titers and IC_{50} values of HBH and NPHBH based on both the homologous and heterologous coating antigens, respectively. The parameters of all antisera obtained from each mouse at the last bleeding are summarized in Table S1. Briefly, all immunized mice showed a significant antibody response to both HBH and NPHBH and an obvious difference of antibody titers and IC_{50} values from these haptens was observed with homologous and heterologous coating antigens (Hapten A, mice antisera analyzed and IC_{50} were detected in the range from 450.4 to 994.2 ng mL^{-1} for HBH, and 359.3–654.3 ng mL^{-1} for NPHBH, Hapten B, 48.3–190.5 ng mL^{-1} for HBH, 35.3–144.1 ng mL^{-1} for NPHBH, Hapten C, 3.9–146.5 ng mL^{-1} for HBH, 2.5–98.7 ng mL^{-1} for NPHBH and Hapten D, 39.5–199.2 ng mL^{-1} for HBH and 33.2–162.7 ng mL^{-1} for NPHBH). In addition, all antisera showed better recognition ability to NPHBH than HBH. Compared to other haptens, antisera derived from Hapten A provided the lowest antibody titers and the highest IC_{50} values for HBH and NPHBH, implying that the frequently used derivative reagent such as 4-FPA for nitrofuran metabolites was exactly not suitable for HBH hapten design perhaps due to the poor stability of the haptens, spacer binding effects or molecular size of the haptens (Diblikova et al., 2005). The antibody titers and IC_{50} values derived from hapten A were almost 10-times and 5-times worse than other haptens when homologous coating antigens were used. However, by using heterologous coating antigens also did not improve the IC_{50} values of antisera to HBH and NPHBH. The antisera obtained from Hapten B, and D showed almost similar antibody titers and IC_{50} values (Table S1). More importantly, in the case of Hapten C, the heterologous coating antigens had significantly improved the IC_{50} values of all antisera for HBH and NPHBH. Particularly, the best IC_{50} values of 3.9 ng mL^{-1} and 2.5 ng mL^{-1} for HBH and NPHBH were obtained from the antisera of Hapten-C-5# while paring with Hapten B-BSA used as coating antigen, that were improved by almost 30 times than those obtained from homologous coating antigen Hapten C-BSA. The antibody titers and IC_{50} values showed that the hapten structure of HBH played an important role in performance of resultant antisera. In the present study, we have introduced three new derivative reagents to prepare haptens with strong immunogenicity after conjugation to protein, particularly in the case of Hapten C. Mouse no. Hapten-C-5# was used to produce monoclonal antibodies because of relatively higher sensitivity.

A total of four hybridomas (2F12, 4D11, 6H6 and 4B12) were prepared as ascetic fluids without further purification. The four mAbs were re-evaluated by using homologous and heterologous coating antigens. As shown in Table S2, the highest affinity, i.e., the lowest IC_{50} values of HBH and NPHBH, were obtained by heterologous coating antigen Hapten B-BSA in case of highly sensitive mAb2F12 (Fig. 3a). Thus, the mAb2F12 and Hapten B-BSA were paired and used in the subsequent experiments. Next, the dilution of antibody and coating antigen were optimized using checkerboard method. It can be observed in Table S3 that the best performance of the icELISA was established with a concentration of coating antigen at 0.42 mg L^{-1} and mAb at 1/27000 dilution.

3.3. Development and optimization of icELISA for HBH

The efficiency of icELISA can be influenced by physicochemical factors related to the buffer (Xu et al., 2016). In order to improve the icELISA performance, some physiological factors were optimized. These factors included: temperature (4 °C, 25 °C, 37 °C), pH values (6.0, 7.0, 7.4, 8.0 and 8.5 for PBS) ionic strength (concentrations of the NaCl were 0.05, 0.1, 0.5 and 1.0 mol L^{-1}) and organic solvent (proportion of ethyl acetate and hexane were 0%, 1%, 5%, 10%). The effect of these factors was determined by measuring the A_{max} and the IC_{50} values. In this study, the A_{max}/IC_{50} ratios were used as the primary criteria to evaluate the icELISA performance and the higher A_{max}/IC_{50} ratio value indicated the higher performance (Liang et al., 2014). A plot of the ratio A_{max}/IC_{50} as a function of temperature is depicted in Fig. 3b. The A_{max} and IC_{50} values were changed when the incubation temperature varied from 4 °C for 24 h, 25 °C for 1 h, and 37 °C for 30 min, and the highest A_{max}/IC_{50} ratio was observed at 37 °C. It was also noticed that the proportion of A_{max}/IC_{50} was the highest at pH 7.4 as shown in Fig. 3c. This finding indicated that a slight difference in pH in the assay buffer apparently affected the icELISA performance. Similarly, impact of the ionic strength on the icELISA performance also showed a similar tendency. The ratio of the A_{max}/IC_{50} was better at ionic strength of 0.1 mol L^{-1} as displayed in Fig. 3d and deviance from this concentration was shown to be detrimental to icELISA performance. These finding indicated that the optimum intensity of the NaCl should not exceed 0.1 M for a desirable sensitivity. In addition, organic solvents are often used in the process of sample pretreatment and excessive organic solvents can result in denaturation of the antibodies and low sensitivity of the icELISA. Therefore, assay must be tested to determine the tolerance of organic solvents. The

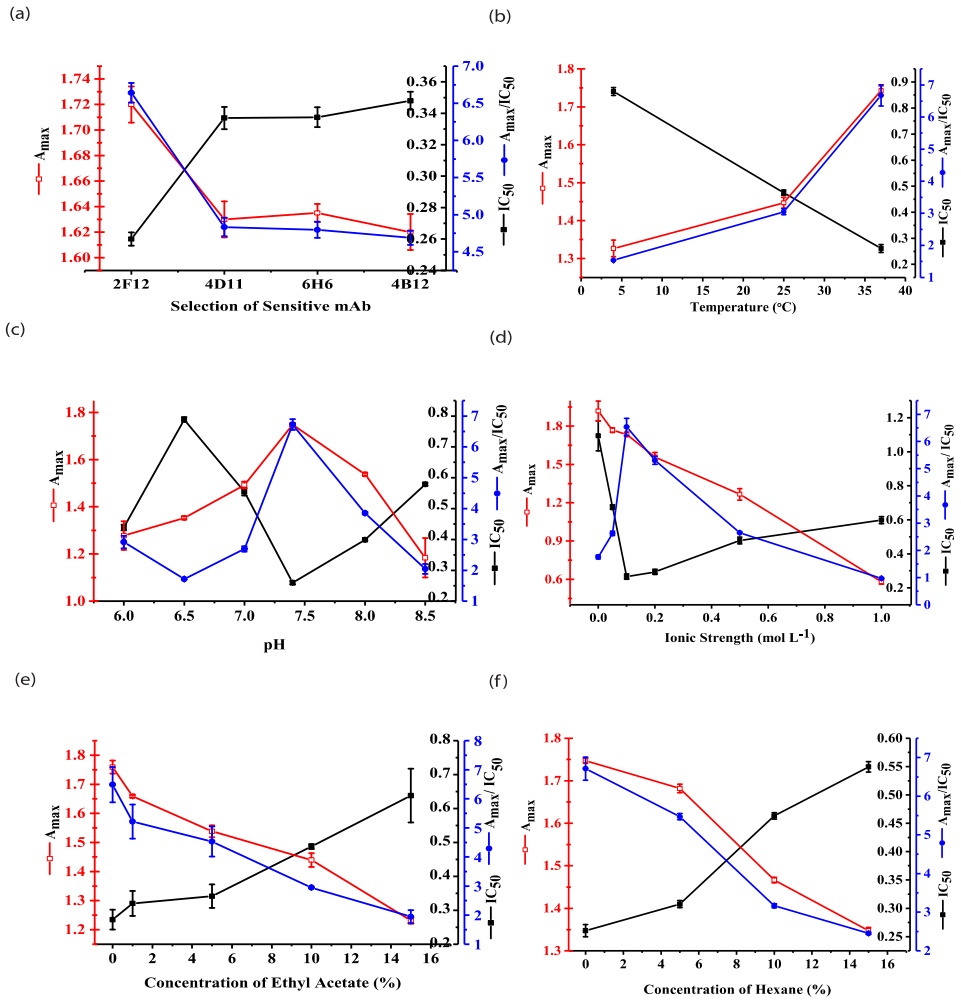


Fig. 3. (a) The selection of sensitive mAb cell line, and the optimization of physicochemical parameters on the icELISA. (b) The effect of Temperature (°C) (c) pH value, (d) ionic strength, (e) ethyl acetate and (f) hexane on the icELISA (n = 3).

ethyl acetate and hexane evaluation were most important to test the performance of icELISA as shown in Fig. 3e and 3f. A noticeable decrease in the A_{max}/IC_{50} values was observed, as the concentration of organic solvent increased.

After optimization of assay conditions, the sensitivity and specificity of the icELISA was determined by conducting competitive assays for the HBH and other analogs (structures shown in Fig. 1). A typical standard curve of the icELISA for HBH, NPHBH and NFX was obtained in Fig. 4a with the IC_{50} values of 0.25, 0.10 and 0.44 ng mL^{-1}, respectively. The limit of detections (LODs) of icELISA in buffer for HBH, NPHBH and NFX, corresponding to the concentration of the standard causing 10% inhibition of tracer binding (IC_{10}), were demonstrated to be 0.04, 0.013 and 0.061 ng mL^{-1}. Particularly, the considerably low LOD values of HBH offered by the mAb2F12-based icELISA can guarantee direct detection of HBH in chicken samples without derivatization step. Similarly, the icELISA developed in the present study can simultaneously detect HBH, NPHBH and NFX since high CRs were obtained with 100%, 250% and 56.81% (Table 1). The specificity of the icELISA was also determined by using other ten compounds including nitrofurans (nitrovin), metabolites (DNSH, AMOZ, AHD, OXZ, SEM, AGD and AOZ)

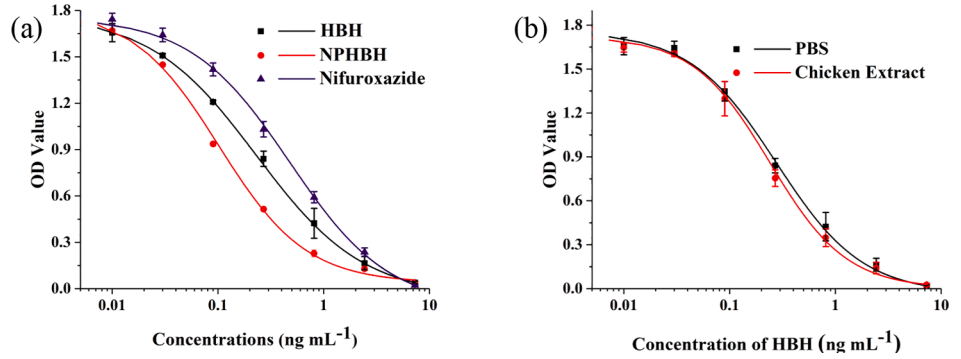


Fig. 4. (a) Standard curves of the icELISA for detection of HBH, NPHBH and Nifuroxazide. (b) The icELISA calibration curves for HBH in PBS and chicken extract. Parallels were found between the standard curve prepared in PBS and chicken extract respectively (N = 3).

Table 1
The IC_{50} values and cross reactivities of the mAb2F12 with HBH and other compounds.

Compounds	IC_{50} (ng mL^{-1})	Cross Reactivity (%)
HBH	0.25	100
NPHBH	0.10	250
NFX	0.44	56.81
2-NBA	>1000	<0.02
4-FPA	>1000	<0.02
DNSH	>1000	<0.02
AMOZ	>1000	<0.02
AHD	>1000	<0.02
OXZ	>1000	<0.02
SEM	>1000	<0.02
AGD	>1000	<0.02
AOZ	>1000	<0.02
Nitrovin	>1000	<0.02

and derivatives (4-FPA and 2-NBA). The values of IC_{50} and the CR are summarized in Table 1. It was also noticed that the icELISA exhibited ignorable CRs with all compounds.

Conformational features and electronic distribution are the objective criteria to understand the recognition mechanism of antigen–antibody. We attempted to explain the possible recognition mechanism of mAb2F12-HBH/NPHBH/NFX in conformational and electronic aspects. As shown in Table 1, mAb2F12 was most sensitive to NPHBH with an IC_{50} of 0.10 ng mL^{-1}. It can be learned that NPHBH possessed the best overlay similarity with Hapten C (Fig. 2a). We supposed that the acetic acid acted as the spacer arm and NPHBH acted as the epitope to elicit antibody towards NPHBH when Hapten C served as the immunizing hapten. Thus, the mAb2F12 displayed the best recognition ability towards NPHBH. Furthermore, the ESP of HBH, NPHBH, NFX were calculated to explain the CR. Compared with NPHBH, the HBH lacked 1-methyl-2-nitrobenzene moiety and possessed strong electronegativity at point B (marked with red arrow in Fig. 2c), which caused approximately 1.5-fold lower recognition ability for HBH-mAb2F12. The point A of NPHBH displayed positive electricity (Fig. 2c), while, the corresponding point C (–NO_2 group) of NFX displayed strong electronegativity, resulting in a 3.4-fold reduction in recognition ability of mAb2F12-NFX. To sum-up, based on above analyses, conformational features and electronic distribution are the key measures to understand the recognition mechanism of antigen–antibody and to improve icELISA performance when the optimum conditions are adopted i.e., pH 7.4 and 0.1 mol L^{-1} NaCl in PBS buffer at 37 °C.

3.4. Matrix effects and recovery

Matrix effect of chicken can hinder an adequate quantification of the HBH. The pretreatment and clean-up are the essential steps for all analytical methods to eliminate matrix effect. Similarly, in this study, chicken samples were spiked with HBH at different concentrations and matrix effects were eliminated by appropriate sample pretreatment steps i.e. acid hydrolysis, solvent extraction using ethyl acetate and finally lipophilic clean-up step with hexane. The extract was collected and the icELISA curve was developed and compared with the standard curve of HBH in buffer as shown in Fig. 4b. The two curves were almost similar, indicating that there was limited interference of matrix to the sensitivity of the icELISA and the method of sample pretreatment was satisfactory. The LOD and detection range of the developed icELISA for HBH in chicken sample was 0.12 μg kg^{-1} and 0.18–1.78 μg kg^{-1}. To evaluate the accuracy of the icELISA for the detection of HBH in chicken, the recovery study was preliminarily investigated. The negative samples were spiked with HBH at four concentrations at 0.5,1.0, 1.5 and 2.0 μg kg^{-1}, respectively. As shown in Table S4, the mean recoveries values ranged from 90.1 to 96.2% with coefficient of variation (CV) values lower than 9.1% following the correction of data by dilution factor of two. These results showed that the icELISA we developed based on mAb2F12 is a potential screening method for the direct detection of HBH in chicken.

4. Conclusion

In the present study, we successfully synthesized haptens of HBH by using new derivative reagents and several high-affinity mAbs were produced with the lowest IC_{50} value of 0.25 ng mL^{-1} for HBH. The best mAb, mAb2F12, showed high CRs with NPHBH and NFX, providing a bright prospect to develop immunoassay for the simultaneous detection of these targets according to individual purpose. A highly sensitive icELISA for the direct detection of HBH was developed with the LOD of 0.12 μg kg^{-1} in chicken without derivatization step. The recovery ranged from 90.1 to 96.2% with CV lower than 9.1%, indicating that the proposed icELISA can be a potential screening method for direct detection of HBH in chicken tissues.

CRediT authorship contribution statement

Ghulam Mujtaba Mari: Conceptualization, Methodology, Writing - original draft, Software. **Hongfang Li:** Data curation. **Baolei Dong:** Methodology. **Huijuan Yang:** Writing - review & editing. **Aisha Talpur:** Data curation, Methodology. **Jiafei Mi:** Methodology, Visualization. **Liuchuan Guo:** Data curation, Methodology. **Xuezhi Yu:** Data curation, Methodology. **Yuebin Ke:** Methodology, Visualization. **Diangang Han:** Methodology, Visualization. **Zhanhui Wang:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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