Highly sensitive immunochromatographic assay for simultaneous determination of azaperone and azaperol in pork

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\textbf{ABSTRACT}

In this study, a sensitive immunochromatographic assay (ICA) was developed for simultaneously detecting azaperone (AZN) and its metabolite azaperol (AZL) based on the high-affinity monoclonal antibody (mAb). Herein, the hapten AZL-SA was synthesized by succinic anhydride method, and then the conjugates AZL-SA-OVA and AZL-SA-KLH were prepared by EDC/NHS method. Subsequently, mAb was produced for targets monitoring through two detection modes. In direct ICA (using gold nanoparticles labeled specific antibodies), the visual LOD of AZN was 80 ng/g. For indirect ICA (using gold nanoparticles labeled anti-species antibodies), the visual and instrumental LODs of AZN were 8 and 0.14 ng/g, and AZL were 8 and 0.12 ng/g, respectively. The results indicated that the visual detection limit (LOD) of indirect format was tenfold lower than that of direct format. The established analytical method obtains the results within 15 min and provides a sensitive and simple tool for on-site detection of AZN and AZL.

1. Introduction

Azaperone (AZN) is a neuroleptic drug belonging to butyrophenones, which is commonly used as sedative for farm animals, especially pigs (Benson & Thurmon, 1979; Taghizadeh, Mohammadnia, Ghalkhani, & Sohouli, 2021). The behavioral inhibition of AZN is mainly mediated by blocking dopamine receptors located in the brain (Osama & Blakely, 2019; Schwarz et al., 2018). The inhibitory effect reduces the ability of animal response towards external signals, which makes them relatively indifferent towards the surrounding environment. AZN is used for treatment of various indications of animals (anesthesia, sedation, stress and anti-aggressiveness) to prevent premature death and loss of meat quality during breeding or transportation (Pohlin et al., 2020; Vitali et al., 2020). However, AZN is usually illegally added for improving fodder conversion rate by reducing animal activity, which can promote weight gain. The accumulation of AZN in edible animal tissues is caused by the improper use of drug dosage or non-observance of withdrawal period (Oliveira et al., 2017), which thus cause damage to the health of consumers through the food chain to result in heart rate reduction, skin vasodilatation, cardiovascular effects and so on (Lin, Wu, Cui, Song, Kuang, & Xu, 2020). It is worth noting that azaperol (AZL), the main metabolite of AZN, can be used as the marker residue to screen AZN (Rauws, Olling, Freudenthal, & Ham, 1976). Moreover, AZL also possesses pharmacological activity. Therefore, so the maximum residual limit (MRL) is regulated to be the sum of AZN and AZL (Stolker et al., 2020). The European Commission stipulates the MRL of AZN and AZL at 100 µg/kg in muscle, skin and fat, kidney and liver of pigs, respectively. In China, the MRL is set to be 60 µg/kg in muscle, skin and fat by the Ministry of Agriculture and Rural Affairs.

In order to monitor AZN residues in animal tissues, multiple analysis techniques have been established, mainly including high-performance liquid chromatography-fluorimetry (HPLC-FL) (Cerkvenik-Flajs, 2007), HPLC with electrochemical detection (HPLC-ECD) (Rose & Shearer, 1992), HPLC with ultraviolet detection (HPLC-UV) (Aoki et al., 2009), gas chromatography-mass spectrometry (GC-MS) (Olmos-Carmona & Hernández-Carrasquilla, 1999), LC-MS/MS (Cooper, Delahaut, Fodey, & Elliott, 2004; Delahaut, Levaux, Eloy, & Dubois, 2003) and high-resolution mass spectrometry (LC-Q-Orbitrap) (Moretti et al., 2018). These instrumental methods are accurate and sensitive. However, they must be conducted under strict conditions, including laborious procedures, highly qualified operator and expensive equipment. In contrast, immunoassay based on high specificity and sensitivity of...
antigen–antibody interaction is considered to be a reliable and simple
detection method, especially immunochromatographic assay (ICA) and
enzyme-linked immunosorbent assay (ELISA) (Zeng et al., xxxx).
However, the application of ELISA for on-site tests was greatly limited by its
repeated processes of incubation, washing and rinsing. ICA has attracted
attention due to its advantages of rapidity, simplicity, economy and
suitability for on-site analysis (Huang et al., 2020; Wang et al., 2021).

Notably, the most important components are molecular recognition
element and the signal transduction element in ICA (Liu, Zhang, Chang,
Zhang, Brennan, & Li, 2015; Majdinasab, Zareian, Zhang, & Li, 2019). At
present, only one literature has reported the detection of AZN by ICA
method (Lin et al., 2020). However, the reported monoclonal antibody
(mAb), a molecular recognition element, was unable to detect AZN and
AZL simultaneously due to its weak cross-reactivity (CR) with AZL. As a
signal transduction element, gold nanoparticle (AuNP) is commonly
applied in the ICA, which has stable performance and low price. Meanwhile,
some other types of materials have been attempted as signal element,
such as fluorescent nanoparticles, magnetic nanoparticles, chemiluminescent
materials, and latex beads (Yang, Xu, & Zhou, 2019; Zvereva, Hendrickson, Zherdev, & Dzantiev, 2020). These materials may improve the sensitivity. However, the complicated preparation
procedure increases the workload and cost. The common mode of ICA
depends on the traditional direct label technology, namely directed
binding between specific antibodies and AuNP. There is also an indirect labeling technique which combines the anti-species antibodies
with AuNP. Compared with the direct ICA, the free specific antibodies of the
indirect ICA have more active sites to bind with the target analytes in
the sample solutions than the antibodies immobilized on the surface of
AuNP because of its faster Brownian motion and higher biological activity
(Byzova, Urusov, Zherdev, & Dzantiev, 2018; Petrokova, Urusov, Gubaydullina, Bartosh, Zherdev, & Dzantiev, 2017). Therefore, the
indirect format of ICA shows a better sensitivity than the direct format
during the detection process. With the above considerations, we designed a novel hapten accord-
ing to the structures of AZN and AZL to produce the mAb with high
affinity which could recognize them at the same time. Subsequently, the indirect format of ICA with high specificity and sensitivity was estab-
lished. This analysis method realized the rapid, sensitive and on-site
detection of AZN and AZL simultaneously, which exhibited good application prospect in actual samples.

2. Material and methods

2.1. Chemicals and apparatus

AZN (purity 99 %), AZL (98 %), xylazine (XYL, 99 %) and carazolol
(CAR, 99 %) were purchased from J&K Scientific Ltd (Beijing, China).
Chlorpromazine (CHL, 99 %) was acquired from Dr. Ehrenstorfer GmbH
(Augsburg, Germany). Droperidol (DRO, 98 %), haloperidol (HAL, 98 %),
succinic anhydride (SA, 99 %) and 4-Dimethylaminopyridine (DMAP, 99 %)
were bought from aladdin (Shanghai, China). Keyhole limpet hemocyanin (KLH, 85 %) and RPMI-1640 were obtained by
Cutter. Mouse myeloma SP2/0 cells were supplied by the British National
Laboratory Animal Center of Zhengzhou University (Zhengzhou, China).
The animal experiments were all approved by the Animal Ethics Com-
mittee and supervised under the guidelines of the Key Laboratory of
Animal Immunization, Henan Academy of Agricultural Sciences. At the
end of the experiment, the slaughter method of animals was carbon
dioxide inhalation.

The Microplate Reader 550 was supplied by Bio-Rad (Richmond,
USA). NanoDrop One was provided by Thermo Fisher (Madison, USA).
XZY Biostrip Dispenser, TSR3000 membrane strip reader and CM4000
Cutter were acquired from Bio-Dot (Richmond, USA).

2.2. Preparation of hapten and antigen

In this work, AZL, the derivative of AZN, was employed to synthe-
sized AZL-SA which was used as the hapten by the succinic anhydride
method. The scheme for the synthesis of AZL-SA was presented in Fig. 1
(a). The processes were briefly described as follows. 6 mg of AZN was
dissolved in 1.5 mL anhydrous pyridine solution. Subsequently, 2.8 mg
of SA and 0.2 mg of DMAP were added. The mixture solution was stirred
and refluxed at 60 °C for 24 h. After the reaction, the solution was dried
by rotary evaporation. Then the sample was redissolved in 600 µL of N,
N-Dimethylformamide. Finally, the obtained hapten AZL-SA was char-
acterized by LC-MS/MS analysis.

AZL-SA-KLH and AZL-SA-OVA conjugates, used as immunogen and
coating antigen, respectively, were prepared using EDC/NHS method by
combination of AZL-SA with KLH and OVA (Chen et al., 2020). The coupling
reaction was conducted at room temperature. 4.0 mg of NHS and
6.6 mg of EDC were appended into the AZL-SA solution and reacted
for 30 min. In addition, 5 mg of KLH and 4 mg of OVA were dissolved in
1 mL of 0.05 M carbonate-buffere saline (CBS, pH 9.6), respectively. 0.25
mL of the activation was slowly added into the KLH solution and incu-
bated for 4 h with continuous stirring to obtain the immunogen. As for
the coating antigen, the remaining activation was added into the OVA
solution under the same conditions. After the incubation, the mixture
was dialyzed with 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 3 d.
The supernatant was collected after centrifuging at 5000 × g for 2 min
and frozen at −20 °C for later use. The AZL-SA-KLH and AZL-SA-OVA con-
jugates were characterized by ultraviolet/visible (UV/Vis) spectroscopy.

2.3. Production of monoclonal antibody against AZN

According to the reported protocol (Sun et al., 2014), four female
BALB/c mice were immunized. The immunogen was AZL-SA-KLH, and
the dose of each immunization was 20 µg with a volume of 200 µL.
The mice were injected for four times at an interval of 21 d. The first
immunogen was emulsified with the same volume of FCA, while FIA was
instead of FCA in the following three immunizations. The indirect
immunogen was emulsified with the same volume of FCA, while FIA was
chosen for the next experiment. The chosen mouse was given
intraperitoneal injection with 40 µg AZL-SA-KLH conjugate. After 72 h,
the spleen cells were fused with SP2/0 cells by PEG 1500. The process of
cell fusion was carried out as previously reported. After that, the fused
cells were dispersed on Ninety-six-well culture plates and cultured in the
incubator at 37 °C with 5 % carbon dioxide. After 8 days, screening
target hybridomas was performed to identify the cell supernatant by
ELISA. Subsequently, the selected cell lines were subcloned using finite
dilution method to obtain the stable antibody-producing clones. The
stable cell line was amplified and intraperitoneally injected into the
mouse. After seven days, the ascites was collected and purified by
octanolic acid-ammonium sulfate method.
2.4. Characterization of mAb against AZN

The titer, specificity, sensitivity, affinity and subtype were used to evaluate the characterization of the mAb against AZN. The subtype determination of the mAb was performed with a MMIK. The titer and affinity were determined by ELISA. ELISA was performed using a standard method that has been reported (Na et al., 2020b). The affinity constant ($K_a$) calculated by Beatty method was identified to evaluate the affinity of the mAb, and the calculation formula was as follows:

$$K_a = \frac{n - 1}{2(n[A_b] - [Ab])}$$

Where, $n$ was the multiple of two different concentrations of coating antigen, $[A_b]$ and $[Ab]$ were the concentration of mAb at 50 % absorbance of the maximum value.

To evaluate the specificity and sensitivity, ic-ELISA was performed by adding a series of standard compounds before addition of antibodies. The 50 % inhibitory concentration ($IC_{50}$) was used to determine the sensitivity, which was defined as the concentration of the standard compounds which inhibited the binding of coating antigen to the antibodies by 50 %. The specificity was assessed by the CRs of AZN and a series of related analytes including AZL, XYL, CAR, CHL, DRO and HAL. The CR was calculated using the following formula:

$$CR(\%) = \frac{IC_{50} of AZN}{IC_{50} of other related analytes} \times 100\%$$

2.5. Preparation of AuNP-labeled anti-AZN mAb and RAGI

Firstly, the preparation of AuNP was carried out by the classical reduction method (Sun et al., 2016). The steps were as follows: 2.0 mL of 1 % (w/v) chloroauric acid was drawn into 198 mL of deionized water and heated and boiled with a rigorous stirring. Then 3.2 mL of 1 % sodium citrate was added into chloroauric acid solution with stirring. It was observed that the color of the mixture changed from gray to red. After boiling and stirring continuously for an additional 5 min, the solution was stopped heating and naturally cooled to room temperature. Finally, the AuNP solution was stored at 4 °C.

The antibody and AuNP were combined by the hydrophobic and electrostatic interactions. The AuNP-labeled mAb was obtained according to the established methodology from literature (Na et al., 2020a). Firstly, the pH of AuNP solution was adjusted to 8.2 by addition of 0.2 M potassium carbonate. For confirming the optimal ratio between antibody and AuNP, 10 µL of the antibody was successively twofold diluted by ultrapure water in microwell. 125 µL of AuNP solution was dropped into each well for incubating 10 min at room temperature, and then 125 µL of 10 % sodium hydroxide liquor was added to terminate the reaction. The optimal ratio between antibody and AuNP was considered as the minimum ration that did not cause the color changes of the mixture. Subsequently, 60 µL of mAb (2.43 mg/mL) was allocated into 20 mL of AuNP liquor and incubated for 2 h at room temperature. After incubation, 1 mL of 20 % BSA was spiked for incubation at 0.5 h to block the residual binding sites on AuNP surface. Next, the resulting deposit was collected with a centrifugation at 12,000 g for 0.5 h. Finally, the AuNP-labeled mAb was dissolved in 10 mL of resuspension buffer (20 mM, pH 9.0) including 0.03 % sodium azide, 3 % sucrose and 1 % BSA, which was stored at 4 °C for further use. The steps of preparing the AuNP-labeled RAGI were the same as above except that 160 µL of GAMI (0.25 mg/mL) was added into 20 mL of the AuNP solution.

2.6. Assembly of the ICA

The ICA device was composed of an adhesive backing board, an absorbent pad (20 cm × 1.6 cm), a nitrocellulose membrane (20 cm × 2.5 cm), a conjugate pad (20 cm × 0.8 cm) and a sample pad (20 cm × 1.6 cm). The absorption pad pulled fluid by capillary force to allow the analyte across the assembly pads. On the nitrocellulose membrane, there was a test (T) line and a control (C) line. The two lines with a 0.45 cm distance were formed by the XYZ Biostrip Dispense at a spraying rate of 0.9 µL/cm, and then dried for 4 h at 42 °C. The sample pad was treated by 0.1 mol/L PBS containing 0.3 % Tween 20, 5 % sucrose, 0.05 % sodium azide and 1 % BSA, and then positioned at 42 °C for 4 h. The conjugate pad was used for receiving recognition labels, which ensured the stability within the warranty period. The recognition labels were sprayed onto the conjugate pad at a rate of 5 µL/cm and laid at 37°C for 1 h. The absorbent pad, nitrocellulose membrane, conjugate pad and sample pad were superimposed on backing board in turn and cut into strips (2.8 mm width) by a CM4000 Cutter.
2.7. Principle of the ICA

According to the principle of competitive immunoassay, two formats (direct and indirect mode) of the ICA were designed. In the direct format, AuNP-labeled anti-AZN mAb was immobilized on the conjugate pad. The T and C line were AZL-SA-OVA and goat anti-mouse IgG, respectively. Generally, the ICA was inserted into the treated sample solution which was successively cross the conjugate pad and membrane towards the absorption pad by capillary force. The results of the sample solution by ICA were presented in Fig. 2(a). In the case of the sample without AZN, the AuNP-labeled anti-AZN mAb was trapped by the coating antigen on the T line to emerge a red line. When AZN presented, AuNP-labeled anti-AZN mAb reacted with the free AZN, which led to less or no combination between it and AZL-SA-OVA. The more target content led to the less AuNP-labeled anti-AZN mAb which would be captured and the weaker color of T line. Regardless of presence or absence of the target, free or coupled AuNP-labeled anti-AZN mAb was always arrested by goat anti-mouse IgG on C line to form a red color, which indicated the effectiveness of the ICA.

In the indirect format, the difference from the direct format was that C line on the nitrocellulose membrane was IgG from mouse and the conjugate pad was the AuNP-labeled GAMI. Before inserting the ICA into the sample solution well, a certain amount of mAb was added and incubated at room temperature for 5 min. The test result was similar to the direct mode, as shown in Fig. 2(b). In the absence of analytes, the mAb would bind to the AuNP-labeled GAMI and was captured by AZL-SA-OVA on T line to generate a red band. In the presence of analytes, the mAb was firstly reacted with the analytes in the sample solution and made fewer mAb binding to the AuNP-labeled GAMI. Therefore, the conjugate compound of mAb and AuNP-labeled GAMI was less intercepted by AZL-SA-OVA to result in a lighter or colorless T line.

2.8. Pretreatment of sample

The pork samples in this study were derived from Henan Academy of Agricultural Sciences, which was previously purchased in the market and determined to be negative by LC-MS. Initially, 5 g of pork was ground into homogenate and transferred into a centrifuge tube (50 mL). Then, 20 mL of acetonitrile was added and vibrated for 2 min, followed by ultrasonic treatment at room temperature for 5 min. The supernatant and precipitate were separated by centrifugation at 5000 × g for 5 min. After collecting the supernatant, 10 mL of acetonitrile was again added and incubated at room temperature for 5 min. The test result was similar to the direct mode, as shown in Fig. 2(b). In the absence of analytes, the mAb would bind to the AuNP-labeled GAMI and was captured by AZL-SA-OVA on T line to generate a red band. In the presence of analytes, the mAb was firstly reacted with the analytes in the sample solution and made fewer mAb binding to the AuNP-labeled GAMI. Therefore, the conjugate compound of mAb and AuNP-labeled GAMI was less intercepted by AZL-SA-OVA to result in a lighter or colorless T line.

2.9. Analysis of the ICA performance

To analyze the ICA performance, a series of concentrations of AZN standard solution were spiked into the treated samples. For the direct ICA, seven concentrations of AZN were selected as 80, 40, 20, 10, 5, 2.5 and 0 ng/g. The concentrations of AZN were 8, 4, 2, 1, 0.5, 0.25 and 0 ng/g in the indirect format. The sensitivity of the ICA in the semi-quantitative test was considered to be the visual detection limit (cutoff value). It was interpreted as the minimum concentration of AZN when T line color disappeared. In addition, the G/D × area of the relative optical density (ROD) values of T line were defined as the quantitative detection and measured using a strip reader. Based on the values decreased when the target concentration increase, a standard curve was obtained. The IC50 and the detection limit (LOD, defined as the target concentration causing 10 % inhibition) were calculated by the standard curve and considered as the sensitivity of the ICA for the quantitative detection. To verify the specificity of the ICA, six related compounds were chosen for the cross reaction.

The recovery experiment was performed by adding three concentrations (0.5, 1, 2 ng/g) of AZN or AZL to the pork samples. Whereafter, T line was detected by a strip reader and the target residues were calculated based on the standard curve. To evaluate the accuracy of the ICA, each sample was determined in triplicate to confirm the coefficient of variation (CV, %).

3. Results and discussion

3.1. Hapten and antigen identification

A small compound <5000 Da cannot directly induce the production of specific antibodies due to lack of T cell epitopes, which is named as hapten without immunogenicity (Wang, Beier, & Shen, 2017). Therefore, small molecules must be attached to carrier proteins in order to generating an immune response. It is well known that the design of hapten and the preparation of antigen are the keys to obtain mAb with high quality. In previous study, Lin et al. prepared the hapten by introducing a linker arm containing four carbon atoms and an active carboxyl group to the benzene ring of AZL, which is then linked with KLH to synthesize the immunogen (Lin et al., 2020). However, the CR value to AZL of the mAb obtained by the hapten was <2.5 % influenced by the mode and location of hapten binding to carrier protein. It indicated that the product could not be used to recognize both AZN and its metabolite AZL.

In this study, we need to consider that the produced mAb could be used to recognize both AZN and its metabolite AZL. Considering that the only difference was a ketone group between AZN and AZL, the hapten was derived by the succinic anhydride method. Then, the hapten AZL-SA was obtained, which could bind to the carrier protein more easily by retaining the common structures to the maximum extent. Simultaneously, the introduction of carboxyl group increased the connecting arm so that...
the specific structure of hapten could show more prominently on the surface of complete antigen. The design of the hapten facilitated the production of highly specific mAb for AZN and AZL. The synthesized derivatives were identified by LC-MS/MS. Fig. 1(b) showed that the m/z [M + H]+ of the main product was 430.2136 for the positive ion analysis. Hence the molecular weight of the compound was 429. It indicated that AZL-SA (C23H28F4N6O4) was successfully synthesized.

The conjugates were initially identified by scanning UV/Vis spectroscopy as shown in Fig. 1(c) and (d). The protein KLH had absorption peaks at 280 nm and 350 nm. Notably, AZL had a strong absorption peak at 238 nm and a weak absorption peak at 300 nm. The synthesized conjugate AZL-SA-KLH not only exhibited a strong absorption peak at 231 nm due to the shift of AZL, but also had the characteristic absorption peak of KLH at 280 nm. The weak absorption of the conjugate at 300–400 nm also reflected the weak absorption peak of AZL at 300 nm and the weak absorption peak of KLH at 350 nm. Meanwhile, the conjugates AZL-SA-OVA and AZL-SA-KLH had similar waveforms. These characteristics indicated that AZN was effectively coupled with KLH/OVA. The formed conjugates could be used as artificial antigens for subsequent experiments.

3.2. Characterization of mAb

A monoclonal cell line 35D1D2 simultaneously identifying AZN and AZL, was produced by cell fusion and hybridoma screening. The titer of mAb represents the absolute number of mAb involved in the antigen–antibody reaction system. The strength of affinity reflects the magnitude of the binding ability between the antigen and the antibody. The K_D value is equilibrium dissociation constant between the antibody and its antigen. According to noncompetitive ELISA analysis, the titer of the mAb was 1.1024 × 10^6 and the affinity was 1.28 × 10^6 L/mol. Based on the ic-ELISA, the sensitivity of the mAb was determined. The concentration of coating antigen and mAb affects the performance of ELISA. In this study, the optimal concentrations of coating antigen and mAb were 0.23 and 0.38 µg/mL, respectively, through microplate method. As shown in Fig. 3(b), the linear equation of standard inhibition curve for AZN and AZL was y = −0.32947x + 0.3508 (R^2 = 0.9916) and y = −0.35707x + 0.31694 (R^2 = 0.9919), respectively. It was worth noting that the IC_{50} of AZN and AZL was 0.35 and 0.31 ng/mL which is lower than that reported (Lin et al., 2020). It indicated that the sensitivity of mAb for AZN was higher than that before. Especially, it can precisely recognize AZL at the same time.

In Fig. 3(a), it revealed that the subclass of mAb from 35D1D2 was IgG2b, and its light chain was Kappa. In addition, the specificity of mAb was estimated based on the CRs of several related analytes by ic-ELISA. The specificity of mAb directly confirms whether the mAb can be applied to complex analysis of real samples. When the CR of mAb is <10%, it can be considered to show no cross-reactivity (Lin et al., 2020). The results were shown in Table 1, the CRs of the mAb with XYL, CAR, CHL, DRO and HAL were all <0.035, indicating the excellent specificity to AZN and AZL. In general, the mAb secreted by 35D1D2 was a suitable antibody for the ICA with satisfactory affinity, specificity and sensitivity.

3.3. Sensitivity of the direct ICA and the indirect ICA

The direct and indirect ICA strips were prepared to detect AZN for comparison. In general, the main parameters affecting the direct ICA are the concentrations of AuNP-labeled mAb and captured antigen. In the direct format, the amounts of AuNP-labeled mAb and AZL-SA-OVA were optimized to achieve the minimum LOD of AZN. After adjusting the color of T line, we found that the jetting rate and the concentration of conjugated AZL-SA-OVA were 6 µL/cm and 0.24 mg/mL, respectively. In the indirect format, the amount of AuNP-labeled GAM1, AZL-SA-OVA and mAb (24.34 µg/mL) was firstly optimized as follows: 6 µL/cm, 0.18 mg/mL, and 3 µL, respectively. The sensitivity of the direct and indirect ICA was shown in Fig. S1. Intuitively, the cutoff value of the direct ICA was 80 ng/g, and indirect ICA was 8 ng/g. It was obviously indicated that the sensitivity was improved by indirect ICA. It may be due to that the free mAb had higher biological activity and longer reaction time between mAb and targets. Compared with the reported ICA, the established indirect ICA is not only 1.25 times more sensitive for the detection of AZN, but also can simultaneously detect its metabolite AZL (Lin et al., 2020). The ICA method allows the detection of total AZN and its metabolite AZL, which can meet the requirements of the European Commission or other countries.

3.4. Analysis of the indirect ICA in pork

The whole experiment process of the indirect format was performed within 15 min including two consecutive stages. In the first stage, the mAb was put into the sample extract to react for 5 min. In the second stage, the ICA was inserted into the extract liquid for testing. The visual image of the ICA was shown in Fig. 4. A series of concentrations of AZN (8, 4, 2, 1, 0.5, 0.25, 0 ng/g) and AZL (8, 4, 2, 1, 0.5, 0.25, 0 ng/g) were added to the sample extract for detecting by indirect format. When 8 ng/g of AZN or AZL were contained in the extracts, the ICA was colorless. Thus, the cutoff values of AZN and AZL for the pork samples were both judged to be 8 ng/g. For the quantitative test, T line was read by a strip reader. Thus, the standard curve was generated by taking the logarithm of target analyte concentration as X axis and the color intensity ratio of T line between spiked sample and non-spiked sample as Y axis. Hence, the equation of standard inhibition curve for AZN and AZL was y = −0.55105x + 0.42786 (R^2 = 0.9972) and y = −0.57486x + 0.3725 (R^2 = 0.98755), respectively. Based on the standard equation, the IC_{50} of AZN was 0.66 ng/g which was slightly less than that of AZL (0.74 ng/g). Meanwhile, the LOD values of them were also acquired, 0.14 ng/g for AZN and 0.12 ng/g for AZL. The working range was defined as the concentration of target analytes that inhibited the corresponding signal by 20 % to 80 % to be 0.21–2.59 ng/g for AZN and 0.18–2.00 ng/g for AZL. The ICA realizes the synchronous detection of AZN and its...
| Compounds | Structures | IC₅₀ (ng/mL) | Cross-reactivity (%) |
|-----------|------------|--------------|----------------------|
| AZN       | ![AZN Structure](image1) | 0.35         | 100                  |
| AZL       | ![AZL Structure](image2) | 0.31         | 112.9                |
| HAL       | ![HAL Structure](image3) | >1000        | <0.035               |
| DRO       | ![DRO Structure](image4) | >1000        | <0.035               |
| XYL       | ![XYL Structure](image5) | >1000        | <0.035               |
| CHL       | ![CHL Structure](image6) | >1000        | <0.035               |
| CAR       | ![CAR Structure](image7) | >1000        | <0.035               |

Table 1
CR of the mAb with other analytes detected by ELISA.
metabolite AZL in pork samples, meeting the detection limits recommended by the European Commission or other countries. The method is a rapid immunoassay, which can be used for semi-quantitative or quantitative determination of AZN and AZL in large quantity of samples.

3.5. Recovery validation

The recovery test was conducted to validate the precision and accuracy of the ICA. AZN and AZL with the concentrations of 0.5, 1, 2 ng/g were spiked into the pork samples, respectively. According to the extraction method in 2.8, the samples were treated for testing with three times repeated. The same batch test strips were used for intra-assay and three different batches were used for inter-assay. Table 2 showed that the recovery rates for AZN and AZL in intra-assay were in the range from 88.12 % to 91.40 %, and the CVs were from 6.13 % to 8.69 %. The recovery rates for AZN and AZL in inter-assay were in the range from 87.61 % to 91.12 %, and the CVs were from 7.16 % to 10.52 %. As is well-known, the recovery rates within 25 % and CVs within 15 % are acceptable (Na et al., 2020a). Consequently, the established ICA was effective and reliable for the determination of AZN and AZL in pork.

4. Conclusions

This study developed an indirect ICA for simultaneously rapid and sensitive detection of AZN and its metabolite AZL. The establishment of this strategy included two key designs: one was to synthesize highly similar hapten AZL-SA through preserving the common structure of AZN and AZL. Subsequently, a satisfactory mAb was produced, which could recognize both of them. The other was to use the indirect format to improve its sensitivity, which allowed mAb to retain high biological activity for combining with the target. The detection process was completed within 15 min, the visual detection limit of AZN and AZL were both 8 ng/g, the IC\textsubscript{50} and LOD values for AZN were 0.74 and 0.14 ng/g, and for AZL were 0.60 and 0.12 ng/g, respectively. The indirect ICA was simple, low-cost and sensitive, and suitable for rapid on-site detection of AZN and AZL in pork. This strategy opens up a facile but powerful avenue of rapid on-site detection platforms for other small molecular targets in food samples.

Statement

All animal experiments were approved by the Animal Ethics Committee and conducted under the guidelines of the Key Laboratory of

### Table 2

| Analyte | Spiked (ng/g) | Tested (ng/g) | Intra-assay | Inter-assay |
|---------|--------------|--------------|-------------|-------------|
|         |              |               | Recovery (%) | CV (%)      | Tested (ng/g) | Recovery (%) | CV (%) |
| AZN     | 0.5          | 0.44 ± 0.03   | 88.12       | 6.13        | 0.44 ± 0.04  | 87.61        | 9.93   |
|         | 1            | 0.91 ± 0.08   | 91.40       | 8.69        | 0.90 ± 0.08  | 89.56        | 9.06   |
|         | 2            | 1.82 ± 0.14   | 91.19       | 7.48        | 1.81 ± 0.13  | 90.46        | 7.16   |
| AZL     | 0.5          | 0.46 ± 0.04   | 91.19       | 8.04        | 0.45 ± 0.04  | 90.39        | 7.79   |
|         | 1            | 0.89 ± 0.06   | 89.10       | 7.28        | 0.90 ± 0.09  | 89.95        | 10.5   |
|         | 2            | 1.81 ± 0.15   | 90.50       | 8.37        | 1.82 ± 0.16  | 91.12        | 8.82   |

CV = coefficient of variation.
Animal Immunization, Henan Academy of Agricultural Sciences. The animals were euthanized by carbon dioxide inhalation after the experiment.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100525.

References

Aoki, Y., Hakamata, H., Igarashi, Y., Uchida, K., Kobayashi, H., Hirayama, N., … Kusu, F. (2009). Simultaneous determination of azaperone and azaperol in animal tissues by HPLC with confirmation by electrospray ionization mass spectrometry. Journal of Chromatography B, 877(3), 166–172.

Benson, G. J., & Thurman, J. C. (1979). Anesthesia of swine under field conditions. Journal of the American Veterinary Medical Association, 174(6), 594-596.

Byzova, N. A., Ursuov, A. E., Zherdev, A. V., & Dzantiev, B. B. (2018). Multiplex highly sensitive immunochromatographic assay based on the use of nonprocessed antisera. Analytical & Bioanalytical Chemistry, 410(7), 1903-1910.

Cerkvenik-Flajs, V. (2007). Determination of residues of azaperone in the kidneys by liquid chromatography with fluorescence detection. Analytica Chimica Acta, 586 (1–2), 374–382.

Chen, L., Sun, Y., Hu, X., Xing, Y., Kwee, S., Na, G., & Zhang, G. (2020). Colloidal gold-based immunochromatographic strip assay for the rapid detection of dimenocin in milk. Food Additives & Contaminants: Part A, 37(1), 1667–1677.

Cooper, J., Delahaut, P., Fodey, T. L., & Elliott, C. T. (2004). Development of a rapid screening test for veterinary sedatives and the beta-blocker carazolol in porcine kidney by ELISA. The Analyst, 129(2), 169–174.

Delahaut, P., Levax, C., Eloy, P., & Dubois, M. (2003). Validation of a method for detecting and quantifying tranquillisers and a β-blocker in pig tissues by liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta, 483(1), 335–340.

Huang, Y., Xu, T., Wang, W., Wen, Y., Li, K., Qian, L., … Liu, G. (2020). Lateral flow biosensors based on the use of micro- and nanomaterials: A review on recent developments. Microchimica acta, 187(10), 7099.

Liu, L., Wei, X., Cui, G., Song, S., Kuang, H., & Xu, C. (2020). Colloidal gold immunochromatographic strip assay for the detection of azaperone in pork and pork liver. ACS Omega, 5(3), 1346–1351.

Liu, M., Zhang, W., Chang, D., Zhang, Q., Brennan, J. D., & Li, Y. (2015). Integrating graphene oxide, functional DNA and nucleic-acid-manipulating strategies for amplified biosensing. TrAC Trends in Analytical Chemistry, 74, 120–129.

Majdinasab, M., Zareian, M., Zhang, Q., & Li, P. (2019). Development of a new format of competitive immunochromatographic assay using secondary antibody–europium nanoparticle conjugates for ultrasensitive and quantitative determination of ochratoxin A. Food Chemistry, 275, 721–729.

Mortelli, S., Lega, F., Rigoni, L., Sahiti, G., Giuseppe, D., Giolito, A., … Galarini, R. (2018). Multiclass screening method to detect more than fifty banned substances in bovine bile and urine. Analytica Chimica Acta, 1032, 56–67.

Na, G., Hu, X., Yang, J., Sun, Y., Kwee, S., Tang, L., … Zhang, G. (2020a). Colloidal gold-based immunochromatographic strip assay for the rapid detection of bacitracin zinc in milk. Food Chemistry, 327, Article 126879.

Na, G., Hu, X., Yang, J., Sun, Y., Kwee, S., Tang, L., … Zhang, G. (2020b). A rapid colloidal gold-based immunochromatographic strip assay for monitoring nitrrozynil in milk. Journal of the Science of Food and Agriculture, 100(5), 1860-1866.

Oliveira, L. G. D., Barreto, F., Hoff, R., Rübensham, G., Kurz, M. H. S., Galle, G., & Gonalves, F. F. (2017). Validation of a method for sedatives and β-blockers determination in swine, bovine and equine kidney using liquid chromatography coupled with tandem mass spectrometry. Food Additives & Contaminants: Part A, 34 (1), 32-39.

Olmos-Canterina, M.-L., & Herandez-Carrasquilla, M. (1999). Gas chromatographic–mass spectrometric analysis of veterinary tranquillizers in urine: Evaluation of method performance. Journal of Chromatography B, 734(1), 113–120.

Osama, R., & Blakely, R. D. (2019). Blockade and reversal of swimming-induced paralysis in C. elegans by the antipsychotic and D2-type dopamine receptor antagonist azapropazone (pp. 59-68). Neurochemistry International.

Petkova, A. V., Ursuov, A. E., Gubaydullina, M. M., Bartosh, A. V., Zherdev, A. V., & Dzantiev, B. B. (2017). “External” antibodies as the simplest tool for sensitive immunochromatographic tests. Talanta, 175, 77–81.

Pohnl, F., Hooijberg, E. H., Buss, P., Huber, N., Vlijmen, F. P., Blackhurst, D., & Meyer, L. C. R. (2020). A comparison of hematological, immunological, and stress responses to capture and transport in wild white rhinoceros bulls (Ceratotherium simum simum) supplemented with azaperone or midaflonam. Frontiers in Veterinary Science, 7.

Rauws, A. G., Oiling, M., Freudenthal, J., & Hanz, M. T. (1976). Azaperol, a new metabolite of the veterinary butyrophenone tranquilizer azapropazone. Toxicology and Applied Pharmacology, 35(2), 333–339.

Rose, M. D., & Shearer, G. (1992). Determination of tranquillisers and carazolol residues in animal tissue using high-performance liquid chromatography with electrochemical detection. Journal of Chromatography, 626(1–2), 471–477.

Schwarz, T., Ziecik, A., Murawski, M., Nowicki, J., Tuz, R., Baker, B., & Bartlewski, P. M. (2018). The influence of azaperone treatment at weaning on reproductive function in sow: Ovarian activity and endocrine profiles during the weaning-to-ovulation interval. Animal, 12(10), 2089–2097.

Stoller, A. A. M., & Birdman, U. A. T. (2005). Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals—a review. Journal of Chromatography A, 1067(1–2), 15–53.

Sun, Y., Hu, X., Zhang, Y., Yang, J., Wang, F., Wang, Y., … Zhang, G. (2014). Development of an immunochromatographic strip test for the rapid detection of zearalenone in corn. Journal of Agricultural and Food Chemistry, 62(46), 11116–11121.

Sun, Y., Xing, G., Yang, J., Wang, F., Deng, R., Zhang, G., … Zhang, Y. (2016). Development of an immunochromatographic strip test for simultaneous qualitative and quantitative detection of oestrone A and zearalene in cereal. Journal of the Science of Food and Agriculture, 96(11), 3673–3678.

Tahzigahed, M. J., Mohammadian, M. S., Ghalkhani, M., & Sohouli, E. (2021). Improved method for the total synthesis of azaperone and investigation of its electrochemical behavior in aqueous solution. Chemical Research in Chinese Universities, 1-6.

Vitali, F., Kärki, E. K., Mijele, D., Kairi, E., Faustini, R., Preziosi, R., … Ravasio, G. (2020). Etorphine-azaperone immobilisation for translocation of free-ranging Masai giraffes (Giraffa camelopardalis tippelskirchii): A pilot study. Animals, 10(2), 322.

Wang, Z., Beier, R. C., & Shen, J. (2017). Immunoassays for detection of macrocyclic lactones in food matrices–A review. Trends in Analytical Chemistry, 92, 42–61.

Wang, Z., Hu, S., Bao, H., Xing, K., Liu, J., Xia, J., … Peng, J. (2021). Immunochromatographic assay based on time-resolved fluorescent nanobeads for the rapid detection of sulfamethazine in egg, honey, and pork. Journal of the Science of Food and Agriculture, 101(2), 684–692.

Yang, H., Xu, W., & Zhou, Y. (2019). Signal amplification in immunoassays by using noble metal nanoparticles: A review. Microchem Acta, 186(12), 859.

Zeng, L., Guo, L., Wang, Z., Xu, K., Song, S., Xu, L., … Xu, C. Immunooassays for the rapid detection of pantothenic acid in pharmaceutical and food products. Food Chemistry, 348, 129114.

Zvereva, E. A., Hendrickson, O. D., Zherdev, A. V., & Dzantiev, B. B. (2020). Immunochromatographic tests for the detection of microcystin-LR toxin in water and fish samples. Analytical Methods, 12(3), 392-400.