Colloidal Gold Immunochromatographic Strip Assay for the Detection of Azaperone in Pork and Pork Liver

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ABSTRACT: In this study, a highly sensitive and specific monoclonal antibody (mAb) against azaperone was produced. The mAb belonged to the IgG2b subtype and its half maximal inhibitory concentration (IC50) was 0.46 ng/mL. The limit of detection value was 0.081 ng/mL and the linear range (IC20−IC80) was 0.165−1.29 ng/mL. Based on this anti-azaperone mAb, a colloidal gold immunochromatographic strip assay method was established for the detection of azaperone in pork and pork liver samples. For pork samples, the cutoff value of the colloidal gold immunochromatographic strip assay was 10 ng/g, and the cutoff value was also 10 ng/g in pig liver samples. This colloidal gold immunochromatographic strip assay is a rapid, simple, and practical method for the detection of azaperone in pork and pork liver samples available in markets.

INTRODUCTION

Azaperone (Figure 1) is an important butylphenol–benzene neurotranquilizer used in the veterinary clinic.† It has several related nerve-stabilizing effects on farm livestock. Intramuscular injection of this drug is used to relieve tension in animals and reduce their activity.2−4 It can make animals indifferent to their environment and keep them in a quiet state for a long term, which is conducive to avoiding fighting when animals live together and in mixed groups. Therefore, it is often used for animals such as pigs during long-distance transportation.5−8 With the widespread application of this drug in veterinary medicine,9 the problem of its residue in animal tissues and the direct harm to human health caused by its toxic side effects have attracted extensive attention. Through the food chain, azaperone still accumulates in the human body, causing a series of adverse reactions such as cardiovascular effects, decreased arterial pressure, skin vasodilation, and heart rate reduction. The maximum residue limits (MRLs) of animal

Figure 1. The chemical structure of azaperone.

Figure 2. The derivative steps of hapten.

Figure 3. (a) Schematic diagrams of the CG immunochromatographic strip; (b) diagram of negative and positive samples.
foods in China stipulate that the MRLs of azaperone and its metabolite azaperol in the muscle, skin and fat, liver, and kidney of pigs are 60, 60, 100 and 100 mg/kg, respectively.

Currently reported detection methods include enzyme-linked immunosorbent assay (ELISA), liquid chromatography (LC)− tandem mass spectrometry (LC−MS), and high performance LC. Although these instrumental methods have high sensitivity and accuracy, they are not suitable for the measurement of large numbers of samples. Compared with the instrumental methods, the colloidal gold (CG) immunochromatographic strip detection method is portable and suitable for on-site detection. In this study, a CG immunochromatographic strip detection method was developed for the detection for azaperone in pork and pork liver.

RESULTS AND DISCUSSION

Verification of Hapten. In order to enable the small molecule to be coupled with carrier protein and possess strong immunogenicity, a linker arm with four carbon atoms and an active carboxyl group were introduced to the side of the hapten. An appropriate length of the linker arm can highlight the characteristic structure of the hapten, which was beneficial to the generation of high-specificity antibodies.

As shown in Figure 4, the mass spectrogram showed a relative mass-to-charge ratio (m/z) of 394, and the relative molecular weight of the corresponding component was 395 (m/z). Because of the negative ion mode, the relative molecular weight of the fragment molecule with a relative molecular weight of 335 (m/z) was consistent with the relative molecular weight of the hapten of Azap, which indicated that the hapten of Azap was successfully derived. The structure of hapten was further confirmed by 1H NMR spectra.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 11.05 (m, 1 H), 8.14−8.13 (m, 1 H), 7.93−7.87 (m, 3 H), 7.38−7.36 (m, 2 H), 7.26−7.23 (m, 1 H), 6.95−6.92 (t, 1 H), 4.47−4.44 (m, 3 H), 3.43−3.41 (m, 2 H), 3.17−3.25 (m, 2 H), 2.70−2.65 (m, 2 H), 2.25−2.10 (m, 2 H), 2.10−2.06 (m, 2 H), 1.86−1.80 (m, 2 H).

Identification of the Immunogen and Coating Antigen. The derived azaperone was an incomplete antigen, which was only antigenic but not immunogenic when present alone. The hapten was not immunogenic until it was coupled with carrier proteins, such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In this study, the hapten Azap was coupled with KLH as an immunogen, and a UV−vis spectrophotometer was used to characterize the conjugates. The UV−vis spectra of hapten, proteins, and conjugates are shown in Figure 5b,c. The hapten Azap had a strong absorption peak at 250 nm and another weak absorption peak at 300−310 nm. The carrier protein KLH had absorption peaks at 278 and 350 nm, and ovalbumin (OVA) had an absorption peak at 278 nm. The conjugate Azap−KLH possessed three characteristic absorption peaks. One of the absorption peaks was at 250 nm, which reflected the hapten. The other two absorption peaks were located at 278 and 350 nm, respectively, which reflected the carrier protein KLH in conjugates. The conjugate Azap−OVA had two absorption peaks at 250 and 278 nm, which were similar to those of Azap hapten and carrier protein OVA. Accordingly, the conjugates of immunogen Azap−KLH and coating antigen Azap−OVA were successfully coupled with carrier proteins.

Characterization of mAbs. The subtypes of mAbs against azaperone are shown in Figure 6a. Obviously, the mAb belonged to the subtype of IgG2b. As shown in Figure 6b, the indirect competitive inhibition curve was drawn with logarithmic values of standard concentrations of azaperone plotted on the abscissa and absorbance values at 450 nm on the ordinate. The equation of the standard curve was obtained, which was $y = 0.0895 \pm 0.1151 + (1.5514 \pm 0.06686−0.0895 \pm 0.1151)/(1 + (x/0.46139 \pm 0.07759))^{1.34972}$. The IC$_{50}$ of the mAb was 0.46 ng/mL, the limit of detection (LOD) value was 0.081 ng/mL, and the linear range (IC$_{20}$−IC$_{80}$) was 0.165−1.29 ng/mL. When the CR rate of a monoclonal antibody (mAb) was less than 10%, it can be regarded as showing no cross-reactivity (CR). As shown in Table 1, the CR data of the mAb against azaperone indicated...
that this mAb was a highly specific mAb because all the CR rates were less than 10%.

| Chemicals | Molecular structure | IC50 (ng/mL) | Cross-reaction rates (%) |
|-----------|---------------------|--------------|--------------------------|
| Azaperone | ![Molecular Structure](image1) | 0.5          | 100                      |
| Azaprool  | ![Molecular Structure](image2) | 20           | <2.5                     |
| Haloperidol | ![Molecular Structure](image3) | >1000        | <0.05                    |
| Spiperone | ![Molecular Structure](image4) | >1000        | <0.05                    |
| Droperidol | ![Molecular Structure](image5) | >1000        | <0.05                    |

**CONCLUSIONS**

In this study, a mAb against azaperone was developed through the interaction of the immunogen and coating antigen. Subsequently, a CG immunochromatographic strip assay was established for the detection of azaperone in different samples. The study confirmed that the CG immunochromatographic strip assay method is feasible for the detection of azaperone in pork and pork liver samples.

**MATERIALS AND METHODS**

**Chemicals.** Azaperone, azaperol, haloperidol, spiperone, droperidol, methyl 4-phenylbutanoate, 4-chlorobutanol chloride, aluminum trichloride, potassium iodide, potassium carbonate, and 1-(pyridin-2-yl) piperazine were purchased from J&K Scientific Ltd. (Beijing, China). The 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-hydroxypyrrrolidine-2,5-dione (NHS), KLH, OVA, carbon disulfide, acetonitrile, tetrahydrofuran (THF), dichloromethane (DCM), N,N-dimethylformamide (DMF), Freund’s complete adjuvant (FCA), Freund’s incomplete adjuvant (FIA), and horseradish peroxidase (HRP)-labelled goat antimus IgG were all purchased from Sigma-Aldrich (St. Louis, MO, USA).
Nitrocellulose (NC) high-flow-plus membrane (Pura-bind RP) was obtained from Whatman-Xinhua Filter Paper Co. (Hangzhou, China). Polyvinylchloride (PVC) backing cards, sample pads (CB-SB08), and absorption pads (SX18) were supplied by Goldbio Tech Co. (Shanghai, China). The coating antigen and mAb against azaperone were obtained from our laboratory.

Mice. All animal studies in this work were performed according to institutional ethical guidelines and were approved by the Committee on Animal Welfare of Jiangnan University. The animal welfare and ethics audit number is JN.No20180930b1001220[205].

Apparatus. The apparatus used for this study was composed of a UV–vis spectrophotometer (Bokin Instruments, Tsushima, Japan), a vortex machine (Shanghai Huxi Analysis Instrument Factory Co. Ltd, Shanghai, China), a water bath (Shanghai Instrument Group Co. Ltd., Supply and Sales Co., Shanghai, China), a Multiskan MKS microplate reader (Thermo LabSystems Inc., Beijing, China), and a membrane dispenser (Xinqidian Gene-Technology Co. Ltd, Beijing, China).

LC–mass spectroscopy (MS) condition as following: Waters 2695-XeVo2 Q-TOF, column C18; column size: 2.1 × 100 mm; mobile phase: from 90% water (0.05% ACS) and 10% CH3CN (0.05% FA) to 5% water (0.05% ACS) and 95% CH3CN (0.05% FA) in 6.0 min, finally under these conditions for 0.5 min.

Synthesis of Hapten. A new type of hapten coupled with structural spacer arms was synthesized based on a previously reported method \(^{15,16}\) (Figure 2). Briefly, methyl 4-phenylbutanoate (1.00 g), 4-chlorobutanol chloride (1.19 g), and aluminum trichloride (1.50 g) were dissolved in 10 mL carbon disulfide. The reaction was warmed to room temperature and stirred overnight. Then, the mixture was poured into ice water, extracted with DCM, dried over Na2SO4 and concentrated to finished, the mixture was cooled to wine red. After continued stirring for 20 min, the color of the boiling solution was then removed until the color of the boiling solution changed to wine red. After continued stirring for 20 min, the reaction was heated at 70 °C overnight. Then, the mixture was cooled to 4 °C until use.

Preparation of CG-Labelled mAbs. CG was synthesized by the trisodium citrate reduction method as reported previously.\(^{26,27}\) In brief, 100 mL of 0.1% chloroauric acid solution was added to a 200 mL conical bottle and placed on an electromagnetic stirring furnace. The solution was stirred and heated to the boiling point. Then, 4 mL of 1% trisodium citrate solution was added to the boiling solution, which was continuously heated and stirred for 15 min. The heat source was then removed until the color of the boiling solution changed to wine red. After continued stirring for 20 min, the solution was cooled to room temperature and the CG solution obtained was stored at 4 °C until use.

Preparation of Immunogen and Coating Antigen. The CG solution was stored at 4 °C and the mAb was precipitated by the octanoic acid-saturated ammonium sulfate precipitation method.\(^{24,25}\) 24,25 The yield (Y, mg/mL) is the amount of antibody extracted per milliliter of ascites (3 mg/mL). After dialysis against PBS for 3 days at 4 °C, mAbs were stored at −20 °C.

Characterization of mAbs. The subtype of the mAb was identified using a mouse mAb isotyping ELISA kit. The IC\(_{50}\) and LOD value of the mAb were used to estimate sensitivity, which was obtained from an indirect competitive inhibition curve. The specificity of the mAb was identified by evaluating its CR with structural analogues of azaperol, haloperidol, spiperone, and droperidol. The indirect competitive inhibition curve of these drugs was drawn by using the same coating antigen and mAb as the standard substance. The cross reaction rates were calculated according to the following formula, CR (%) = (IC\(_{50}\) of Azap mAb/IC\(_{50}\) of structural analogues) × 100%.

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Under conditions in which the isoelectric point of immunoglobulin (mAb) was slightly alkaline, the CG was adjusted to pH 7.0 and constantly stirred overnight at room temperature to finish the conjugation of hapten and CG. The Azap–CG conjugate was dialyzed against PBS for 48 h with a solution change every 8 h to remove uncoupled hapten, then stored at −20 °C until use. According to Lambert–Beer’s law, the coupling rate of small molecular hapten with CG was 78. The approach to coating antigens was as described above. The conjugates were verified by ultraviolet spectroscopy.

Production of mAb Against Azaperone. Ten female BALB/c mice (6–8 weeks of age) were immunized with the immunogen (Azap–KLH).\(^{18}\) The mice were immunized by subcutaneous injection of the antigen and FCA after emulsification.\(^{19,20}\) FCA was used for the first immunization (100 µg per mouse) and FIA was used for the booster immunization (50 µg per mouse).\(^{21,22}\) Antiserum titer and inhibition were screened by ic-ELISA. The mouse with the highest titer which showed competitive inhibition with azaperone was selected for cell fusion. Twenty-five microgram of immunogen was injected intraperitoneally into the mouse in the first three days of cell fusion.

Mouse spleen cells and myeloma cells were fused using polyethylene glycol.\(^{23}\) The titer of cells was detected by ic-ELISA. The inhibitory effect of a positive cell line on the target drug was further determined by ic-ELISA. The cell line with the best inhibitory activity was subcloned three times by the limited dilution method. Ultimately, a mAb hybridoma cell line against azaperone was screened. Hybridoma cell lines were injected into the abdominal cavity of mice to produce ascites, and the mAb was purified by the octanoic acid-saturated ammonium sulfate precipitation method.\(^{24,25}\) The yield (Y, mg/mL) is the amount of antibody extracted per milliliter of ascites, and in this study, the yield was 3 mg antibody extracted per milliliter of ascites (3 mg/mL). After dialysis against PBS for 3 days at 4 °C, mAbs were stored at −20 °C.

Characterization of mAbs. The subtype of the mAb was identified using a mouse mAb isotyping ELISA kit. The IC\(_{50}\) and LOD value of the mAb were used to estimate sensitivity, which was obtained from an indirect competitive inhibition curve. The specificity of the mAb was identified by evaluating its CR with structural analogues of azaperol, haloperidol, spiperone, and droperidol. The indirect competitive inhibition curve of these drugs was drawn by using the same coating antigen and mAb as the standard substance. The cross reaction rates were calculated according to the following formula, CR (%) = (IC\(_{50}\) of Azap mAb/IC\(_{50}\) of structural analogues) × 100%.
was adjusted to pH 8.0 with 0.1 M K₂CO₃ and 8 μg of mAb against azaperone was added. The mixture was stirred at room temperature for 50 min and mixed with 1 mL of 10% (w/v) BSA solution for occlusion and stabilization. The solution was stirred for 1 h at room temperature, and centrifuged at 10 000 g for 30 min at 4 °C. The supernatant was discarded, while the pellet was resuspended in 2 mL of 10 mM resuspension buffer (20 mM pH 8.2 Tris, 0.1% PEG, 0.1% Tween, 5% sucrose, 5% trehalose, and 0.2% BSA) three times. Finally, the pellet was resuspended in 1 mL of the same resuspension buffer and stored at 4 °C until use.

**Assembly of the CG Immunochromatographic Strip.** A schematic diagram showing the CG immunochromatographic strip is presented in Figure 3a. Based on the PVC backing card, an NC membrane used as the carrier was located in the center of the PVC backing card. The absorption pad and the sample pad were attached to either end of the PVC backing card, and the gold pad was located between the sample pad and the NC membrane, overlapping the NC membrane or the strip card, and the gold pad was located between the sample pad and 0.2% Tween 20 and dried at 37 °C until use.

**Detection Procedure and Principle.** The sample pad was immersed in the liquid sample solution and incubated at room temperature for 5 min, and the mixed liquid migrated slowly toward the absorption pad through capillary action. Because of the color reaction of CG, the results could be observed with the naked eye in 5–8 min.

The principle of a CG immunochromatographic strip is presented in Figure 3b. In negative samples, the CG-labelled mAb shifts to the absorption pad with the solution, and then specifically binds to the coating antigen on the T line and the goat antimouse IgG on the C line. It can then be observed that both T and C lines turn red. In positive samples, the CG-labelled mAb binds with the target object. As the number of target objects increases, the amount of uncoupled CG-labelled mAb decreases, resulting in a lighter T line color.

**Sample Pretreatment and Analysis.** Azaperone is a neuroleptic, which is commonly used in pigs. Consequently pork and pork liver were purchased from a local supermarket as samples for detection. Appropriate adjustments had been made to the methods reported in the references to the pretreatment process. Briefly, 2.00 g homogenized pork was weighed and put into a 50 mL centrifuge tube, then mixed with 10 mL acetonitrile. The mixture was whirled, oscillated at 824g for 5 min and ultrasonicated for 2 min at room temperature. The insoluble material and supernatant were isolated by centrifugation at 4750g for 5 min, and then 10 mL acetonitrile was added to the residue for repeated extraction. The supernatant obtained from the two extractions were merged together. The supernatant was dried under nitrogen in a water bath at 50 °C. Subsequently, the extract was redissolved in 2 mL PBS for analysis.

The samples were spiked with different concentrations of azaperone before extraction, and tested using the CG immunochromatographic strip assay.

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**Notes**

The authors declare no competing financial interest.

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