Enzyme-Linked Immunosorbent Assay and Immunochromatographic Strip for Rapid Detection of Atrazine in Three Medicinal Herbal Roots

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Abstract

Objectives: An enzyme-linked immunosorbent assay (ELISA) and colloidal gold-based immunochromatographic (ICG) strip assay will be developed for the rapid and high-throughput detection of atrazine (ATZ) in medicinal herbs. Methods: A monoclonal antibody against ATZ was obtained after the immunization of mice, cell fusion, and hybridoma screening, and the antibody was used to develop direct competitive ELISA (dcELISA) and the ICG strip assay. Results: Both dcELISA and ICG strip methods were established, optimized, and validated for the detection of ATZ in Salviae miltiorrhizae radix et rhizome, Astragali radix, and Isatidis radix. dcELISA had a half-maximum inhibition concentration of 10.56 ng/mL (Salviae miltiorrhizae radix et rhizome), 7.6 ng/mL (Astragali radix), and 8.15 ng/mL (Isatidis radix). The limit of detection (LOD) of the ICG strip assay was 12.5 ng/mL (Salviae miltiorrhizae radix et rhizome), 12.5 ng/mL (Astragali radix), and 6.25 ng/mL (Isatidis radix) in different herb matrices. Due to the recognition characteristics of the monoclonal antibody for the pesticides ATZ, propazine, sebuthylazine, and prometryn, the detection results of real samples by the two immunoassays were confirmed by liquid chromatography–tandem mass spectrometry, which proved the accuracy and reliability of the established methods. Conclusion: The proposed dcELISA and ICG strip methods were suitable for the rapid, convenient, and high-throughput detection of ATZ in these medicinal herbs.

Keywords: Astragali radix, atrazine, isatidis radix, monoclonal antibody, salviae miltiorrhizae radix et rhizome

Introduction

Atrazine (ATZ) is one of the most broadly used triazine herbicides, which was utilized to control broadleaf weeds. Because ATZ is cheap and selective, its widespread use has led to an extensive contamination of soil, water, and agriculture products.[1] Moreover, due to its high mobility, persistence, and low degradation rate, ATZ has become the most commonly detected herbicide in environmental resources. Several reports have confirmed that exposure to ATZ could induce severe diseases in humans such as physiological disorders, low blood pressure, muscle spasms, and adrenal gland damage.[2-4] Therefore, the residual levels of ATZ need to be monitored.

Currently, many methods have been established for the detection of ATZ residues in environmental samples,[5] foodstuffs,[6] and agricultural products.[7,8] Due to their sufficient accuracy and high sensitivity, liquid chromatography (LC), LC–tandem mass spectrometry (LC-MS/MS), gas chromatography (GC), and GC–tandem mass spectrometry (GC-MS/MS) are the most commonly used methods for detecting ATZ.[6,9] However, these instrumental analysis methods usually require

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highly trained personnel, expensive instruments, complex pretreatment of samples, and time consuming. These methods are not suitable for on-site screening of ATZ. Efforts have been made to establish highly selective and rapid detection methods for the screening of a large number of samples. Immunoassays can meet the requirements for the detection of ATZ because of their specific recognition of antigens and antibodies. Based on the principle of immunoassays, many novel methods have been reported for the rapid detection of ATZ such as enzyme-linked immunosorbent assay (ELISA),[10,11] time-resolved fluorescent immunoassay,[12] colloidal gold-based immunochromatographic (ICG) strip assay,[13,14] electrochemical immuno sensors,[11] and fluorescence immunoassay.[15] Among them, ELISA is the most widely used method in immunoassay analysis because of its low cost, simple operation, and ability of high-throughput detection of samples.

As special agricultural products, medicinal herbs are also easily contaminated by ATZ. ATZ may be prone to enter the soil and groundwater due to its water solubility properties, and this increases the probability of contaminating the roots of medicinal herbs. Several studies have reported that ATZ was more easily detected in medicinal herbs, the medicinal part of which is the root.[16–18] However, there have been no reports on the detection of ATZ in medicinal herbs using ELISA and ICG strips. Therefore, it is meaningful to establish immunoassay methods for the rapid detection of ATZ in medicinal herbs.

In this study, a monoclonal antibody of ATZ was prepared and used to develop direct competitive ELISA (dcELISA) and ICG strip assays for the detection of ATZ residues in herbs. The proposed methods had acceptable stability and sensitivity; thus, they can be applied to detect ATZ residues in Salviae miltiorrhizae radix et rhizome, Astragali radix, and Isatidis radix. The detection results of real samples were consistent with the LC-MS/MS measurements, which confirmed the reliability and practicality of these established methods.

**Experiment**

**Materials and reagents**

Triazine standards (ATZ, propazine, sebutylazine, terbution, prometryn, terbutylazine, simazine, ATZ-desethyl, ATZ-desisopropyl, and ATZ-desethyl-desisopropyl), complete and incomplete Freund’s adjuvant, N-hydroxysuccinimide, bovine serum albumin (BSA), ovalbumin (OVA), hors eradish peroxidase (HRP), hypoxanthine aminopterin thymidine, hypoxanthine thymidine, and N, N-dicyc lohexylcarbodiimide were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse Sp2/0-Ag14 myeloma cell line was purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China). Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from Gibco BRL (Paisley, UK). Nitrocellulose (NC) membranes, glass fiber membranes, sample pads, absorbent paper, and poly (vinyl chloride) sheets were obtained from Jieyi Biotech Shanghai Co. (Shanghai, China). The other analytical reagents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Chromatographic-grade reagents (methanol and formic acid) were purchased from Honeywell China Co. (Shanghai, China).

**Preparation of antigen, mAb, and enzyme tracer**

The structures of the two haptens have been reported in a previous study. All the antigens were prepared using the active ester method, as shown in Figure 1. Hapten-1 and hapten-2 were covalently attached to BSA and OVA, respectively. Hapten-1-BSA was used to immunize Balb/c mice, and hapten-2-OVA was used as the coating antigen for the ELISA and ICG strip assay. The procedures for generating the immune response in female Balb/c mice and producing mAbs were the same as in our previous report. Briefly, female Balb/c mice (6–8 weeks old) were immunized with the emulsified immunogen and Freund’s adjuvant at 2-week intervals. After the third immunization, the titer and specificity of the serum were tested, and the mouse with the highest specificity and highest titer was selected for the preparation of hybridoma cells. The polyethylene glycol (PEG) method was used to fuse splenocytes and Sp2/0-Ag14 cells. Further selection through icELISA and the hybridomas positive for ATZ were cloned. The mAbs were obtained from the resulting ascites and were purified according to the ammonium sulfate precipitation method. Using the active ester method, the enzyme tracer (ATZ-HRP) was prepared by combining hapten-1 with HRP.

**Sample preparation**

The three root medicinal herbs were purchased from a medicinal material market. Salviae miltiorrhizae radix et rhizome, Astragali radix, and Isatidis radix were finely ground, homogenized, and passed through an 80 mesh (0.18 mm) sieve. A 3.0 g sample was weighed and placed into a 50 mL centrifuge tube, 10 mL of 75% methanol was added into the tube, and vortexed for 5 min. Then, the tube was subjected to ultrasonic extraction for 15 min. Samples were centrifuged at 5000 rpm for 15 min. Finally, the obtained supernatant was filtered through a 0.22 µm nylon syringe filter, and this filtrate was used as an analytical solution for LC-MS/MS. For dcELISA, the extracted supernatant was diluted 10 times using a sample diluting solvent (0.01 M phosphate-buffered saline [PBS] containing 0.1% Tween-20 and 0.1% gelatin, pH 7.4). For the ICG strip assay, 1 mL of the extracted supernatant was dried under nitrogen and re-dissolved with 1 mL of sample diluting solvent.

**Direct competitive enzyme-linked immunosorbent assay**

The microplate wells were coated with the prepared mAb diluted in carbonate-buffered saline (100 µL) at 4°C overnight. The unbound antibodies were washed with PBST (PBS containing 0.1% Tween 20) four times. Standard or sample solutions were then diluted with the sample diluting solvent, added into the wells, and incubated together with 50 µL of the enzyme tracer. After incubation at room temperature for 30 min, the microplate was washed four times and subsequently incubated.
with 100 μL of substrate solution for 15–20 min. The color reaction was stopped by adding 100 μL of 2 M H₂SO₄ to each well. Finally, the absorbance values were measured at 450 nm using a microplate reader.

**Preparation of immunochromatographic strip and immunochromatographic strip assay**

The preparation and characterization of colloidal gold particles was reported in our previous study. The process of synthesizing colloidal gold–antibody conjugate was as follows: the pH of the colloidal gold solution (12 mL) was adjusted by adding 72 μL of 0.2 M K₂CO₃, and 72 μg of mAb was added drop-wise with stirring. The mixture was incubated with stirring for 30 min. Then, 1% BSA (600 μL) was used to block the residual surface of the colloidal gold, and the block process required 30 min of incubation. The colloidal gold–antibody conjugate precipitate was obtained by centrifugation at 10,000 rpm for 30 min. Finally, the conjugate precipitate was dissolved in a resuspension buffer (PBS containing 1% BSA, 0.1% Tween 20, 5% sucrose, and 0.2% PEG 20,000).

The colloidal gold–antibody conjugate was sprayed on a glass fiber membrane at 5 μL/cm and dried at 37°C. The test line (T line) (hapten-2-OVA, 1 mg/mL) and control line (C line) (goat anti-mouse IgG, 1 mg/mL) were separately coated on the NC membrane at 1 μL/cm and dried at 37°C. The assembled ICG strips were then cut into 4 mm wide strips. When the ICG strip was used for the test, 80 μL of the sample solution was added drop-wise onto the sample pad. After 5–10 min of color reaction, the result was obtained by the naked eye, based on the color change. The color change of a positive sample was evidenced by the disappearance of the T line, while the C line remained visible.

**Liquid chromatography–tandem mass spectrometry verification**

LC-MS/MS was used for the validation studies. LC separation was achieved using Shimadzu ultra-fast LC (Kyoto, Japan) with a Waters Acquity UPLC BEH Shield RP 18 column (50 mm × 2.1 mm, 1.7 μm). The flow rate was set at 0.20 mL/min. The column temperature was maintained at 40°C. The mobile phase consisted of methanol (a) and aqueous formic acid (0.1%, v/v) (b); the gradient elution process was as follows: 70% B at 0–3 min, 70%–5% B at 3–8 min, and 5%–70% B at 8–12 min. An AB SCIEX QTRAP® 5500 mass spectrometer (Foster City, CA, USA) was used for ATZ quantitation. It was ionized in the positive ion mode and analyzed by LC-MS/MS with the following parameters: curtain gas, nebulizer gas, and auxiliary gas were set at 35, 55, and 55 psi, respectively; the ion spray voltage was 5500 V; the declustering potential was 100 V and 20 V, and the source temperature was 550°C. The precursor ion

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**Figure 1:** Preparation procedure of antigens. BSA: bovine serum albumin, OVA: Ovalbumin, DCC: N, N’-dicyclohexylcarbodiimide.
was m/z = 216.1 (M + H)⁺ and the monitoring ions were m/z = 216.1 > 95.9 and 216.1 > 104.0. m/z = 216.1 > 95.9 was selected as the quantitative ion. The collision energies were set at 20 V and 38 V, respectively.

**Results and Discussion**

**Characterization of the antigens**

The prepared antigens were confirmed by ultraviolet (UV) absorption. The UV absorbance spectra are shown in Figure 2. The results showed that the antigens had adsorption peaks of the corresponding hapten and protein, which indicated that the antigens were successfully obtained.

**Development of direct competitive enzyme-linked immunosorbent assay**

**Optimization of direct competitive enzyme-linked immunosorbent assay**

The diluted concentrations of mAb (1 mg/mL) and ATZ-HRP (1 mg/mL) were optimized using two-dimensional checkerboard titration. The dilution series of mAb and ATZ-HRP were 1:100, 1:300, 1:900, 1:2700, 1:8100, 1:24,300 and 1:100, 1:300, 1:900, and 1:2700, respectively. Referring to the analysis step of dcELISA, the optimal working concentrations of mAb and ATZ-HRP were selected corresponding to an absorbance value close to 1.0. Finally, the optimal diluted concentrations of both mAb and ATZ-HRP were 1:900, and these parameters were used in dcELISA.

**Evaluation of direct competitive enzyme-linked immunosorbent assay**

To reduce matrix interference, a series of matrix-matched standards were prepared using a blank matrix extract solution to obtain the inhibition curves [Figure 3]. The calculated half-maximum inhibition concentration values in different matrices were 9.4 ng/mL (PBS), 10.56 ng/mL (Salviae miltiorrhizae radix et rhizome), 7.6 ng/mL (Astragali radix), and 8.15 ng/mL (Isatidis radix), which indicated that there was a slightly enhanced effect on the antigen–antibody interaction and the sufficient sensitivity of the mAb. Moreover, the linearity ranges were 2.2–50.9 ng/mL (PBS), 2.4–45.7 ng/mL (Salviae miltiorrhizae radix et rhizome), 1.6–26.3 ng/mL (Astragali radix), and 1.4–48.9 ng/mL (Isatidis radix), which were based on the IC20 and IC80 inhibitory concentrations of the inhibition curve.

The accuracy of the proposed dcELISA method was also evaluated. The three herb samples were spiked with a certain amount of ATZ (0.15, 0.3, and 0.6 mg/kg) and their

| Table 1: Recoveries of atrazine in spiked herbs by direct competitive enzyme-linked immunosorbent assay and liquid chromatography–tandem mass spectrometry |
|-----------------|-----------------|-----------------|-----------------|
| Herb            | Spiked concentration (mg/kg) | Recovery (%)±SD | dcELISA | LC-MS/MS |
| Salviae miltiorrhizae radix et rhizome | 0.15 | 80±3.2 | 84±5.7 |
| Astragali radix | 0.3 | 84±4.6 | 86±7.7 |
| 0.6 | 86±2.1 | 82±5.4 |
| Isatidis radix | 0.15 | 85±2.5 | 93±3.4 |
| 0.3 | 84±5.1 | 93±3.3 |
| 0.6 | 93±1.2 | 83±3.3 |
| 0.15 | 85±3.4 | 90±2.1 |
| 0.3 | 83±4.7 | 110±1.4 |
| 0.6 | 91±1.1 | 86±3.6 |

ELISA: Enzyme-linked immunosorbent assay, dcELISA: Direct competitive ELISA, SD: Standard deviation, LC-MS/MS: Liquid chromatography–tandem mass spectrometry

Figure 2: Ultraviolet absorbance spectrums of haptens, bovine serum albumin, ovalbumin, and antigens

Figure 3: Inhibition curves for atrazine in different herb matrices
recoveries were calculated. For comparison, the recovery of the LC-MS/MS method was also conducted at the same spiked concentrations. The results [Table 1] proved that the accuracy of the established dcELISA method is highly consistent with that of the LC-MS/MS method.

**Cross reactivity**

For evaluating cross-reactivity, nine triazine herbicides with a structure similar to ATZ were used. The structures of the ten triazine herbicides and their cross-reactivity results are shown in Table 2. It can be seen that propazine (123.6%), sebuthylazine (83.9%), and prometryn (74.0%) had a relatively higher cross-reactivity with the mAb compared with that of the other herbicides, and these compounds have similar structures to ATZ. ATZ-desethyl (DEA), ATZ-desisopropyl (DIA), and ATZ-desethyl-desisopropyl (DEDIA) had low cross-reactivity with the mAb, which demonstrated that both the nitrogen substituent structures had high interaction abilities with the mAb. In addition, simazine showed only a slight cross-reactivity with the mAb, which revealed that the isopropylamino group could serve as an immunodominant group for this hapten. However, sebuthylazine, terbutylazine, and terbumeton without an isopropylamino group but with different alkyl substituents in the same part exhibited significantly different cross-reactivity values, which proved that the structure of the alkyl substituent also had a certain effect on the cross-reactivity. Owing to the recognition characteristics of monoclonal antibodies, the established rapid detection method can have a better monitoring effect on ATZ, propazine, sebuthylazine, and prometryn simultaneously. The other triazine herbicides cannot influence the performance of the immunoassay. For the positive sample, the detection result should be confirmed by performing LC-MS/MS.

| Table 2: Cross-reactivity of atrazine and other triazine herbicides |
|---|
| **Analytes** | **Chemical structure** | **IC$_{50}$ (ng/mL)** | **Cross-reactivity (%)** |
| Atrazine | | 9.4 | 100 |
| Propazine | | 7.6 | 123.6 |
| Sebuthylazine | | 11.2 | 83.9 |
| Prometryn | | 12.7 | 74.0 |
| Terbuthylazine | | 50.9 | 18.5 |
| Terbumeton | | 61.3 | 15.3 |
| Simazine | | 83.0 | 11.3 |
| Atrazine-desethyl (DEA) | | 76.2 | 12.3 |
| Atrazine-desisopropyl (DIA) | | 803.2 | 1.2 |
| Atrazine-desethyl-desisopropyl (DEDIA) | | - | - |

**Development of the immunochromatographic strip assay**

**Optimization of the immunochromatographic strip assay**

The preparation of the colloidal gold conjugate was optimized by adjusting the quantity of 0.2 M K$_2$CO$_3$ and mAb (1 mg/mL). First, different quantities of 0.2 M K$_2$CO$_3$ (2, 4, 6, and 8 μL) and mAb (2, 4, 6, and 8 μg) were added to 1 mL colloidal gold solution, and 100 μL 10% NaCl was added to it. When the color of the colloidal gold solution changed to blue, it indicated that the preparation failed. Finally, the quantities of K$_2$CO$_3$ and mAb added were 6 μL/mL and 6 μg/mL, respectively. The concentrations of the T and C lines were also investigated, and the result was judged by the color change. The optimal concentration for the T and C lines was 1 mg/mL.

**Evaluation of the immunochromatographic strip assay**

The detection limit is defined as the concentration of ATZ in the solution that causes the T line to disappear. In this study, several concentrations of ATZ standard (0, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 ng/mL) were prepared in PBS and herb matrix solution to investigate the sensitivity of the ICG strip assay. As shown in Figure 4, the detection limits of ATZ in PBS, Salviae miltiorrhizae radix et rhizome, Isatidis radix, and Astragali radix were 12.5 ng/mL, 12.5 ng/mL, 6.25 ng/mL, and 12.5 ng/mL, respectively, proving that matrix interference was low, which was consistent with the dcELISA method.
The stability and specificity of the ICG strip assay were also evaluated. The prepared ICG strips could still be used for ATZ detection when stored at 4°C, 37°C, and room temperature for 1 month, indicating that the strips had excellent stability. The cross-reactivity results also revealed that propazine, sebuthylazine, and prometryn had high cross-reactivity with the mAb, which was in agreement with the dcELISA results.

**Real sample analysis**

The established dcELISA and ICG strip assays were used to detect residual ATZ in 14 real herbal samples. The detection results were verified by LC-MS/MS. As shown in Table 3, residual ATZ (0.08 mg/kg) was detected in a batch of Salviae miltiorrhizae radix et rhizome samples, and the quantitative result was consistent with that of LC-MS/MS. In addition, a positive result (+) was also obtained by the strip assay in the same sample. The results proved the accuracy and reliability of the established dcELISA method for detecting ATZ residue. Although the ICG strip assay could not be used for accurate quantification, it could achieve rapid on-site screening of ATZ residues.

In previous studies, a variety of immunoassay methods have been established for ATZ. We collected and compared the existing detection methods, applicable matrix, and limit of quantitation (LOD), and their results are shown in Table 4. The currently established analytical methods used for the detection of ATZ are focused on foods, soil, and water. Although the detection methods are more diverse, their application in a complex herbal matrix remains questionable. Compared with cereals and water matrices, the immunoassay method designed for herbs is usually more complicated. For this reason, the LODs in this work were slightly higher than in other matrices, but they were sufficient to control triazine herbicides. In addition, a simple extraction and dilution method for complex sample pretreatment was established and applied in this work, which greatly reduces the cost of pretreatment. In summary, the proposed ELISA method can accurately evaluate the pollution levels of ATZ, and the establishment of an ICG strip can screen the presence of ATZ herbicide pollution in the field.

**Conclusion**

In this study, an ATZ mAb was prepared and used to establish a sufficiently accurate and sensitive dcELISA method for ATZ residue detection in different root medicinal herbs (Salviae miltiorrhizae radix et rhizome, Astragali radix, and Isatidis radix) and water. The results proved the accuracy and reliability of the established dcELISA method for detecting ATZ residue. Although the ICG strip assay could not be used for accurate quantification, it could achieve rapid on-site screening of ATZ residues.
Multilayer graphene oxide-based paper for the detection of ATZ residues in medicinal herbs. This study also provides research value for the detection of ATZ residues in other medicinal herbs.

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There are no conflicts of interest.

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Conflicts of interest

There are no conflicts of interest.

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