High sensitivity and specificity Enzyme-Linked immunosorbent assay for detection of Acetaminophen in herbal tea and liquor

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Abstract. The acidified structure of acetaminophen (ACA) and 3-Oxo-3-(phenylamino)propanoic acid (3-O-3) were used to couple bovine serum albumin (BSA) to prepare haptens, respectively. A highly sensitive and specific competitive indirect enzyme-linked immunosorbent assay (ciELISA) method was developed for the detection of acetaminophen using 3-O-3-BSA for New Zealand white rabbit immunization to produce antibodies. The IC50 was 6.66 ng/mL, the detection limit was 0.32 ng/mL, and the quantitative detection range was 0.91-48.54 ng/mL. Comparing the performance of ACA-BSA antibody and 3-O-3-BSA antibody recognition of acetaminophen, it was found that the carbonyl site of 3-O-3 plays a major role in antigen-antibody recognition, while para-phenolic hydroxyl does not play an important role in the recognition process. The study provided theoretical guidance for the preparation of acetaminophen recognition antibodies, and established a rapid detection method for acetaminophen.

1. Introduction

Acetaminophen is a metabolite of phenacetin in the body, which can be used to treat cold, fever and postoperative pain [1-3]. Acetaminophen can replace part of the function of aspirin for patients who are allergic to aspirin [4-5]. However, acetaminophen should not be used in excess, and continuous use should not exceed 3 days. It mainly damages the human liver and can cause death in severe cases [6-9]. In 2017, the World Health Organization (WHO) has listed acetaminophen as a three-category carcinogen. At present, many companies still use acetaminophen to treat cold, fever and postoperative pain. It is illegally added to herbal tea and even white wine. Acetaminophen brings great safety hazards to consumers. However, conventional large-scale instrument analysis has the characteristics of long cycle and high cost. Therefore, it is necessary to develop a fast and convenient method for acetaminophen detection.

At present, the detection methods for acetaminophen are mostly concentrated in thin layer chromatography, high performance liquid chromatography. These methods require professional technicians and necessary equipment and are not suitable for on-site large-scale screening. The enzyme-linked immuno sorbent assay (ELISA) has the advantages of high throughput, high sensitivity, and fast reaction speed, and is suitable for on-site batch determination [10-13]. However, this technology relies on the performance of antibodies. The reported preparation methods of acetaminophen detection antibodies are relatively random, and lack of prediction on the performance
of the prepared antibodies, which is a serious waste of manpower and material resources [14-17].

In this study, the two structures of acetaminophen acidified structure (ACA) and 3-oxo-3-(phenylamino) propionic acid (3-O-3) were used to prepare corresponding antibodies respectively. The ability of two antibody to recognize acetaminophen was compared. The key recognition sites of the acetaminophen recognition antibody were initially analyzed. It lays the foundation for the preparation of high-sensitivity antibodies for the detection of acetaminophen and the development of high-sensitivity detection methods. This article established a rapid detection method for acetaminophen, which can specifically identify acetaminophen.

2. Materials and methods

2.1. Materials and apparatus

Tween-20, complete and incomplete Freund’s adjuvant, N-Hydroxysuccinimide (NHS), 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide (EDC), ovalbumin (OVA), bovine serum albumin (BSA), anti-Rabbit IgG-Biotin antibody produced in goat (GaR IgG-HRP) were purchased from Sigma-Aldrich (St. Louis, MO). ACA, 3-O-3, aflatoxin and melamine were purchased from Veterinary Medicine Supervisory Institute of China (Beijing, China). Phosphate buffer solutions (PBS) of various pH was adjusted by mixing 1/15M stock solutions of KH$_2$PO$_4$ and Na$_2$HPO$_4$ at different ratios, while the washing buffer in the immunoassay was PBST by adding 0.5% tween-20 in 0.01 M PBS 7.4.

ELISA absorbance values were measured at a wavelength of 450 nm with an iMark microplate reader (Bio-rad). ELISA plates were washed in a Wellwash MK2 microplate washer (Thermo Scientific). Ultraviolet-visible (UV-vis) spectroscopy was detected on a UV-4000 spectrophotometer (Hitachi).

2.2. Preparation of immunogens and coating antigens

Dissolve NHS (1 mg), EDC (1 mg) and ACA (1 mg) in 0.5 mL DMF. Dissolve NHS (1 mg), EDC (1 mg) and 3-O-3 (1 mg) in 0.5 mL DMF. The above two reaction mixtures were stirred at 4°C for 12 hours. BSA (10 mg) and OVA (10 mg) were dissolved in 1 mL of 0.01 M carbonate buffer. The ACA activation solution (0.5 mL) and 3-O-3 activation solution (0.5 mL) were added dropwise to the protein solution (BSA, OVA). The mixture was stirred for 3 hours. Finally, the reaction product was dialyzed against 0.9% (m/v) NaCl solution at 4°C for 3 days, and stored at -20°C until use. The haptens prepared by this method are called ACA-BSA and 3-O-3-BSA, and the coating antigens prepared are called ACA-OVA and 3-O-3-OVA, and characterized by UV-vis spectroscopy [18-20].

2.3. Preparation of polyclonal antibodies

Three 12-week-old New Zealand white rabbits were subcutaneously immunized with 325 μL Freund’s complete adjuvant and 325 μg ACA-BSA or 3-O-3-BSA mixed emulsification. Three weeks after the initial injection, emulsify with the same amount of immunogen and incomplete Freund’s adjuvant, and give booster injections to New Zealand white rabbits, and booster immunization every three weeks. A small amount of rabbit blood was drawn for testing one week after the third immunization to observe the immunization effect. The polyclonal antibody was collected ten days after the fourth immunization and stored at -20°C until use [21-22].

2.4. Enzyme-linked immunosorbent assay

The 96-well plate was coated overnight with the coating antigen (100 μL/well) in carbonate buffer at 37°C. Wash the 96-well plate twice with PBST (300 μL/well) (phosphate buffered saline solution containing 0.1% Tween-20). Seal with 5% skimmed milk powder (120 μL/well) at 37°C for 3 hours. The 96-well plate was dried at 37°C for 1 hour. Add the diluted paracetamol standard solution (50 μL/well) and the diluted antibody (50 μL/well) to a 96-well plate. After incubating in a 37°C water bath for 40 minutes, the 96-well plate was washed 5 times with 300 μL PBST. Then add 100 μL/well of HRP-IgG (diluted 1:5000 in PBST), and incubate the 96-well plate at 37°C for 30 minutes. After
washing five times with 300 μL PBST, add 100 μL TMB solution to each well (400 μL 0.6% TMB-dimethyl sulfoxide and 100 μL 1% H2O2 are diluted with 25 mL pH 5.5 citric acid-acetate buffer). Incubate it at 37°C for 10 minutes. Finally, add 50 μL 2 M H2SO4 to stop the reaction. Use a microplate reader to record the absorbance of the reaction solution at 450nm (A450) [23].

2.5. Statistical analysis and curve fitting
A series of acetaminophen standard solutions of different concentrations were mixed with the antibody for testing. The absorbance value was plotted against the analyte concentration to obtain a standard curve. Use Origin 8.5 software (Origin Lab Corp., Northampton, MA) to draw S-shaped curve with parametric equation:

\[ Y = \frac{(A - D)}{[1 + (x/C)^B]} + D \]  \hspace{1cm} \text{Eq. 1}

A and D correspond to the maximum and minimum absorbance, respectively. B is the slope of the curve, and C is the concentration of acetaminophen that inhibits antibody binding by 50% [24-25].

2.6. Cross-reactivity
The specificity of the ELISA method was identified by comparing the cross-reactivity of other compounds with acetaminophen. The cross-reactivities (CR) formula is as follows:

\[ \text{CR} = \frac{\text{IC}_{50} \text{ (acetaminophen)}}{\text{IC}_{50} \text{ (other compounds)}} \times 100\% \]  \hspace{1cm} \text{Eq. 2}

where IC50 refers to the concentration that inhibits 50% of antibody binding [26-27].

2.7. Actual sample detection
Acetaminophen was blended into herbal tea and white wine, and formulated into three concentrations of 2 ng/mL, 8 ng/mL, and 16 ng/mL, respectively. Use the ELISA method to simultaneously detect the sample to be tested and the standard product of the same concentration, do three parallel tests for each sample, compare the test results, and observe the sample recovery rate.

3. Results and discussion
3.1. Preparation of artificial antigen
The UV-visible spectra of hapten, coating antigens, BSA, OVA, ACA and 3-O-3 are shown in Figure 1. BSA and OVA showed absorption peaks at 280 nm that were absent from ACA and 3-O-3. The coupling of ACA and 3-O-3 to the carrier protein resulted in significant changes in the characteristic peaks of the hapten and the coating source, and had an absorption peak at 280 nm, and the absorption characterization chart Inconsistent with BSA and OVA. Therefore, it is proved that the carrier protein is successfully coupled with ACA and 3-O-3. The structure of ACA and 3-O-3 is shown in Figure 2.

![Fig. 1. UV-vis spectra of artificial antigens, BSA, OVA, ACA and 3-O-3](image-url)
3.2. Standard curve of acetaminophen

ELISA reaction shows the antibody prepared by ACA-BSA inhibited the recognition of 1 μg/mL acetaminophen by 19% (Figure 3). Inhibition of recognition of 1 μg/mL acetaminophen by the antibody prepared by 3-O-3-BSA was 92%. Therefore, the antibody prepared by 3-O-3-BSA was used to establish the standard curve of acetaminophen.

Comparing the structure of ACA and 3-O-3, it is found that the carbon chain of 3-O-3 is longer than ACA. ACA may be small in structure and not immunogenic, so the antibody prepared by ACA-BSA has no obvious recognition effect on acetaminophen. The structure of 3-O-3 has no phenolic hydroxyl structure at the para position, but the antibody prepared by 3-O-3-BSA still has a good effect on acetaminophen. The recognition ability indicates that the phenolic hydroxyl structure may not play a major role in antigen and antibody recognition. 3-O-3 has one more carbonyl structure than ACA. The extra structure allows the antibody prepared by 3-O-3-BSA to effectively recognize the Acetaminophen indicates that the carbonyl site is the main site recognized by the antigen and antibody. The structure of this site of ACA is blocked due to the coupling of the carrier protein, so the antibody prepared by ACA-BSA is not as capable of recognizing acetaminophen as the antibody prepared by 3-O-3-BSA.

ACA-OVA was used for coating antigens to establish a standard curve for the detection of acetaminophen (Figure 4). The IC_{50} was 6.66 ng/mL, the detection limit was 0.32 ng/mL, and the quantitative detection range was 0.91-48.54 ng/mL.
3.3. Method-specific testing
As shown in Table 1, except for acetaminophen, the cross-reactions of other compounds to acetaminophen are all less than 0.1%. Therefore, it shows that the antibody prepared by 3-O-3-BSA has no obvious cross-reaction to other harmful drugs in herbal tea and has good specificity.

Table 1 Cross-reactivity of acetaminophen

| Molecules     | IC₅₀ (ng/mL) | CR (%) | LOD (ng/mL) |
|---------------|--------------|--------|-------------|
| Acetaminophen | 6.66         | 100.0  | 0.32        |
| Aflatoxin     | >1000        | <0.1   | -           |
| Melamine      | >1000        | <0.1   | -           |

3.4. Method stability testing
As shown in Table 2, the recovery rate of acetaminophen in liquor is 92%-98%, and the recovery rate of acetaminophen in herbal tea is 100%-104%. The recovery rate of the actual sample test and the standard test is very close, and the CV value is less than 8%. It shows that the developed ELISA method can be applied to the detection of actual samples with good stability.

Table 2 Recovery rate of actual sample detection

| Sample     | Add (ng/mL) | Mean concentration (ng/mL) | Recovery (%) | CV (%) |
|------------|-------------|----------------------------|--------------|--------|
| herbal tea | 2.0         | 2.05±0.06                  | 102.5        | 2.9    |
| herbal tea | 8.0         | 8.07±0.29                  | 100.8        | 3.6    |
| herbal tea | 16.0        | 16.51±0.42                 | 103.2        | 2.5    |
4. Conclusions

Acetaminophen is used for the treatment of colds and fever, postoperative pain relief, and can replace part of the aspirin function. However, it is listed as a three-category carcinogen by the WHO, so it should not be used in excess, and continuous use should not exceed 3 days, otherwise it will cause damage to the human liver and even cause death. Because of its certain effects, many food companies have illegally added acetaminophen to herbal tea or even white wine, which brings great safety hazards to consumers. This study established a detection method for acetaminophen, with a minimum detection concentration of 0.32 ng/mL.

The reported detection methods for acetaminophen include GC/MS and HPLC. These methods require professional technicians and necessary valuable equipment and are not suitable for large-scale rapid screening on site. The ELISA method developed in this research is convenient, fast, and highly specific, suitable for on-site high-throughput sample screening, and the detection limit of the method is 0.32 ng/mL. The detection limit is in the same order of magnitude as the high performance liquid chromatography developed by Jing Zan et al. [28].

At present, the sensitivity IC\textsubscript{50} of the ELISA method developed in this research reaches 6.66 ng/mL, which is one of the most sensitive ELISA methods reported so far.

Combined with the structure of acetaminophen, two structures of ACA and 3-O-3 were designed and synthesized for the first time, and corresponding antibodies were prepared respectively. Comparing the ability of antibodies to recognize acetaminophen, combining the two structural differences, it is found that ACA may be small in structure and not immunogenic, and the prepared antibody has no obvious recognition effect on acetaminophen. 3-O-3-BSA antibody has a good ability to recognize acetaminophen, but 3-O-3 has no phenolic hydroxyl structure in the para position, while acetaminophen has position phenolic hydroxyl, indicating that the phenolic hydroxyl structure may not play a major role in antigen and antibody recognition. 3-O-3 has one more carbonyl structure than ACA, while 3-O-3 antibody has a good recognition effect on acetaminophen, indicating that the carbonyl site is the main site recognized by the antigen antibody. And the structure of this site of ACA is blocked due to the coupling of the carrier protein. Subsequent research can prepare the corresponding crystal structure of the ACA-BSA antibody and 3-O-3-BSA antibody. Combining crystal analysis, comparing the above conclusions, this study accurately analyzing the effective recognition sites of acetaminophen, and providing materials for the study of antigen-antibody recognition mechanism.

In this study, the two structures of ACA and 3-O-3 were used to prepare corresponding antibodies respectively. The performance of antibodies to recognize acetaminophen and combining the two structural differences, the results showed that the carbonyl site of 3-O-3 plays a key role in the body's antigen and antibody recognition, while the para-phenolic hydroxyl group not play a key role in the process. This research result provides a scientific basis and practical guidance for the preparation of acetaminophen recognition antibody. It also provides a theoretical basis for establishing a rapid detection method that specifically recognizes acetaminophen in food safety. The established rapid detection method exhibits its characteristics of high sensitivity and strong specificity. It can quickly screen out the test objects of acetaminophen that exceed the national standard. It can effectively eliminate false positive test results during the detection process.
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Notes and references
There are no conflicts of interest to declare.

Appendix A. Supporting information
There is no supporting information.

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