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One-step ultra-sensitive immunochromatographic strip authenticating an emergent fraud acetophenetidin in herbal tea

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Abstract

Herbal tea is highly popular and widely consumed beverage in Lingnan area, China. However, a pain-relieving and fever-reducing drug, acetophenetidin, was recently found to illegally occur in herbal tea on a fraud purpose. Due to the potential health risk and urgent requirement for on-site screening method, a one-step simple strip for identifying acetophenetidin was developed for the first time.. Assisted by computational chemistry, four haptens were designed to prepare immunogens and coating antigens for antibody generation, and a specific antibody with ultra-sensitivity was generated, showing half maximal inhibitory (IC_{50}) of 16.46 ng/mL for acetophenetidin, less than 3.5% of cross-reactivity to analogs. Molecular modeling revealed that opposite electrostatic potential on the immunizing hapten surface to that of the analyte contributed the sensitivity of the resultant antibody. A gold nanoparticles immunochromatographic strip was developed for one-step detection of acetophenetidin in herbal tea, demonstrating an estimated cut-off value of 160 ng/mL, a quantitative limit of detection of 1.63 ng/mL, and recoveries ranging from 102.1% to 106.1%. The analysis of spiked (n=?) and real samples (n=20) by the strip was well correlated with that of the confirmatory method, liquid chromatography−tandem mass spectrometry. The proposed strip has the potential to be used for rapid screening of acetophenetidin in Chinese herbal tea.

Keywords: acetophenetidin, hapten, antibody, strip, immunochromatography, herbal tea

1. Introduction

Acetophenetidin (APD, $C_{10}H_{13}NO_2$, N-(4-ethoxyphenyl) acetamide) (**Figure 1**), is a non-steroidal anti-inflammatory drug, originally as an analgesic and antipyretic medicine (Ghasempour, Dehestani, & Hosseini, 2020). Its adverse effects reported on health included methemoglobinemia, sulfhemoglobinemia, even damage kidney and induce cancer (Salles, Araujo, & Paixão, 2016; Yin, Meng, Xu, Chen, & Ai, 2012). The U.S. Food and Drug Administration (US-FDA) banned the use of pharmaceutical preparations containing acetophenetidin in 1983 (Salles, et al., 2016), and it was also not listed in "Chinese Pharmacopoeia". Moreover, the International Agency for Research on Cancer has listed acetophenetidin as a Class I carcinogen (Landwehr, Larcombe, Reid, & Mullins, 2021). Acetophenetidin was associated with an increased risk of death due to urologic or renal diseases, death due to cancers, and death due to cardiovascular diseases (Dubach, Rosner, & Stürmer, 1991). In addition, people with glucose-6-phosphate dehydrogenase deficiency may experience acute hemolysis, or dissolution of blood cells, while taking this drug. Acute hemolysis is possible in the case of patients who develop an IgM response to phenacetin leading to immune complexes that bind to erythrocytes in blood. The erythrocytes are then lysed when the complexes activate the complement system. It is believed that the metabolite acetophenetidin is at least partly responsible for these effects (Kankuri, Solatunturi, & Vapaatalo, 2003).

Guided by health preserving theory of traditional Chinese medicine, on the base of herbal properties and local climate and the characteristics of water and earth, Chinese in Lingnan area (mainly located in Guangdong, Guangxi, Hongkong, Macau) through the years developed a hot beverage called herbal tea that it is believed to the efficacy of “cleaning internal heat, detoxification and stopping thirst”. Herbal tea is brewed from

the leaves, flowers, seeds, fruits, stems or roots of medicine (Zhao, Lv, Chen, & Li, 2011) and it is still popular nowadays. However, acetophenetidin was recently found to illegally occur in herbal tea intentionally added to enhance its efficacy. Our of 456 retail batches of herbal tea tested, 3 batches were found containing acetophenetidin in concentrations from 49~520 mg/mL (He, Wen, Lai, & Cao, 2018).

Currently, the detection methods for acetophenetidin mainly includes high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Schelstraete, Devreese, & Croubels, 2018), ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Zhe Wang, et al., 2019), gas chromatography mass spectrometry (GC-MS) (Fiorentin, Logan, Martin, Browne, & Rieders, 2020). These instrumental analysis methods exhibited good sensitivity, accuracy and reliability. However, they require expensive equipment, complex sample preparation, well-trained personnel, which limits their applications in the on-site rapid screening assay of acetophenetidin. Immunoassay, known as rapid, low cost, sensitive method was established based on the specific antigen-antibody binding theory. Among them, later flow immunochromatographic strip is proven simple to operate, and fast, with the test result ready in 5-20 minutes. Nevertheless, there has not been any immunochromatographic assay reported in the literature for acetophenetidin detection.

In this study, four kinds of haptens were rationally designed by aligning the common structure and the molecular electrostatic potential surfaces for antibody generation using computational methods based on molecular modeling. After obtaining an antibody with ultra-sensitivity, a one-step gold nanoparticles (GNPs) immunochromatographic strip was developed for the rapid assay of acetophenetidin in herbal tea. The sensitivity, specificity, accuracy and precision were also evaluated and verified, and real retail samples were analysed by both the novel

immunochromatographic strip and reference LC/MS-MS method to confirm the reliability of the proposed methodology

2. Materials and methods

2.1 Reagents and animals

Acetophenetidin, aminopyrine, chlorpheniramine, aminopyrine, metronidazole, phenylbutazone, salicylic acid, piroxicam, ibuprofen, diclofenac sodium, dexamethasone, dexamethasone acetate, ethyl 4-bromobutyrate, methyl 4-(bromomethyl)benzoate, methyl 4-(bromomethyl)benzoate, ethyl bromoacetate, and 3,3',5,5'-tetramethylbenzidine (TMB), and acetone were purchased Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), bovine serum albumin, ovalbumin, Freund's complete adjuvant, Freund's incomplete adjuvant, peroxidase-labeled goat anti-rabbit antibody IgG, and goat anti-rabbit antibody IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). N, N-Dimethylformamide (DMF), Tween-20 and methanol were obtained from Damao Chemical Reagent Factory (Tianjin, China). Tetrachloroauric(III) acid tetrahydrate (CAS: 16903-35-8) was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane (Sartorius, UniSart CN95, Goettingen, Germany) was purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). The polyvinylchloride (PVC) backing plate (SMA31-40), sample pad (SB08), and absorbent pad were obtained from Shanghai Kinbio Tech. Co., Ltd. (Shanghai, China). Other chemicals were purchased from Guangzhou Chemical Reagent Co., Ltd (Guangdong, China). All reagents were of analytical reagent grade or higher purity.

New Zealand white rabbits 2−3 months old (about 2-3 kg) were sourced from Guangdong Medical Experimental Animal Centre and raised at the Animal Experiment Centre of South China Agriculture University (Animal Experiment Ethical Approval Number: 2019141). All the necessary licenses to work with animals were secured prior to the commencement of the work.

2.2 Instruments

A NanoDrop2000c spectrophotometer, MK3 microplate reader was supplied by Thermo Fisher China (Shanghai, China), The XYZ^{TM} Dispense Platform comprised motion control with Biostrip Dispenser HGS102 and Airjet HGS102 was purchased from BioDot Inc (Irvine, CA, USA). Programmable Sheet Cutter and Programmable strip cutter were purchased from Shanghai Kinbio Tech. Co., Ltd. (Shanghai, China). The GL-23M Centrifuge was provided by Xiangyi Centrifuge Instrument Co., Ltd. (Changsha, China). The Q100N test strips reader was obtained from Suzhou Hemai Precision Instrument Co., Ltd.

2.3 Hapten synthesis

The synthesis scheme of the hapten 1−4 is shown in **Figure 1,** and each hapten was identified by mass spectrometry and nuclear magnetic resonance spectroscopy (NMR).

2.3.1 Hapten 1: 4-(4-acetamidophenoxy)butanoic acid

Acetaminophen (1 mol) and potassium carbonate (4 mol) were dissolved in 5 mL of acetone, and ethyl 4-bromobutyrate (1.1 mol) was added to the above solution with stirring for 3 h at room temperature. The above reaction product was purified to obtain ethyl 4-(4-acetamidophenoxy) butanoate. Then, this was dissolved in methanol, and NaOH solution (1 M) was added with stirring for 3h at room temperature. After the

reaction was completed, the pH value of the solution was adjusted to ~~approximately~~ 7.0 with HCl solution (1 M) and Hapten 1 or 4-(4-acetamidophenoxy)butanoic acid was recovered. The following results were obtained from the ESI-MS analysis (negative): m/z 236.50 $[M-H]^-$, ^1H NMR (600 MHz, Methanol-d4) δ 7.44 – 7.39 (m, 7H), 6.90 – 6.84 (m, 7H), 4.00 (t, J = 6.2 Hz, 7H), 2.49 (t, J = 7.3 Hz, 7H), 2.10 (s, 10H), 2.09 – 2.01 (m, 8H), 1.30 (s, 3H), 1.25 (t, J = 7.1 Hz, 1H), 0.94 – 0.85 (m, 2H).

2.3.2 Hapten 2: 4-((4-acetamidophenoxy)methyl)benzoic acid

The same synthesis procedure was used for hapten 2 as for hapten 1, except that methyl 4-(bromomethyl)benzoate was used instrad of ethyl 4-bromobutyrate. The following results were obtained when analysing the final reaction product 4-((4-acetamidophenoxy)methyl)benzoic acid: ESI-MS analysis (negative): m/z 283.90 $[M-H]^-$, and ^1H NMR (600 MHz, Methanol-d_4) δ 8.07 – 7.98 (m, 11H), 7.55 (dp, J = 7.6, 0.8 Hz, 11H), 7.48 – 7.41 (m, 11H), 7.00 – 6.94 (m, 11H), 5.15 (s, 11H), 4.69 (s, 1H), 3.91 (s, 1H), 2.10 (s, 16H), 1.95 (s, 3H), 1.31 (d, J = 19.5 Hz, 2H).

2.3.3 Hapten 3: 2-(4-acetamidophenoxy)acetic acid

The same synthesis procedure used for hapten 1was also used for hapten 3 , except that ethyl bromoacetate was used instead of ethyl 4-bromobutyrate. The following results were obtained when analysing the final reaction product *: 2-(4-acetamidophenoxy)acetic acid* : ESI-MS analysis (negative): m/z 207.9 $[M-H]^-$, and ^1H NMR (600 MHz, Methanol-d_4) δ 7.43 (d, J = 8.7 Hz, 1H), 6.90 (d, J = 9.0 Hz, 1H), 4.40 (s, 1H), 2.11 (s, 1H).

2.3.4 Hapten 4: 2-(4-acetamidophenoxy)propanoic acid

The same synthesis procedure used for hapten 1 was also used for hapten 4, except that ethyl 2-bromopropionate was used instead of ethyl 4-bromobutyrate. The following

results were obtained when analysing the final reaction product 2-(4-acetamidophenoxy)propanoic acid. ESI-MS analysis (negative): m/z 223.2 [M-H]-,and ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.47 – 7.40 (m, 4H), 6.91 – 6.83 (m, 4H), 5.51 (s, 1H), 4.77 (q, J = 6.8 Hz, 5H), 4.12 (q, J = 7.2 Hz, 15H), 3.37 (s, 1H), 2.11 (s, 6H), 2.03 (s, 23H), 1.96 (s, 1H), 1.58 (d, J = 6.8 Hz, 7H), 1.37 – 1.29 (m, 5H), 1.26 (t, J = 7.1 Hz, 24H), 0.95 – 0.87 (m, 2H).

2.4 Preparation of immunogen and coating antigen

The hapten-1 and hapten-2 were coupled to carried protein BSA and OVA to obtain six conjugates for the use of immunogens (hapten-1-BSA, hapten-2-BSA) and coating antigen 1-4 (1, hapten-1-OVA. 2, hapten-2-OVA. 3, hapten-3-OVA. 4, hapten-4-OVA), prepared according to an active ester method (~~Y.~~ Zhang, et al., 2017), respectively.

2.5 Generation of antibody

The generation of rabbit polyclonal antibody (pAb) was carried out according to previous work (Y. Zhang, et al., 2017). To select the optimal combination of antibody and antigen for high performance, the titer and inhibition rate (1-B/B_0) was used to characterize the binding ability of antibody with antigen and analyte respectively, where B and B_0 represent the absorbance values of the acetophenetidin standard solution and negative solution, respectively (Y. Zhang, et al., 2017; Zou, et al., 2016). Then, the calibration curve was established by plotting B/B_0 against the concentration of acetophenetidin, and the half maximal inhibitory (IC_{50}) was calculated. To characterize the specificity (expressed as cross-reactivity, CR) of the antibody, other drugs for the CR were determined and calculated according to the following equation (Xie, et al., 2022):

$$CR\ (\%) = IC_{50(analyte,\ ng/mL)} / IC_{50(analogs,\ ng/mL)} \times 100$$

2.6 Molecular Modeling

The molecular modeling was carried out according to the previous work (Xie, et al., 2022). Firstly, the structures of acetophenetidin, acetaminophen and the four haptens were sketched into the SYBYL-X 2.1.1 (Tripos Inc.) software. These structures were geometry optimized to global low energy conformations using the standard Tripos force field with an 8 Å cutoff for nonbonded interactions in conjunction with Gasteiger-Hückel charges. The criteria of the termination and max iterations were set at 0.005 kcal/(mol × Å) and 10000. The dielectric constant was set to 1.00. The surface electrostatic potential of acetophenetidin, acetaminophen and of the haptens were obtained by the MOLCAD surface algorithm of SYBYL-X.

2.7 Antibody labeling

All glassware was washed with aqua regia and dried, 182 mL of ultra-purified water and 8 mL of chloroauric acid solution (1%, w/v) were heated to boiling under constant stirring, then 10 mL of trisodium citrate solution (1%, w/v) was immediately fast added, the solution color gradually changed from light yellow to purple red. The reaction was boiled for another 15 min after the solution color had changed to purple red, and was finally left to cool to room temperature and stored at 4°C for further use.

For the labeling of antibody with GNPs, the procedure was similar to previous work (Li, et al., 2019). Briefly, 1 mL of GNPs solution was added to a centrifuge tube and pH -adjusted with K_2CO_3 solution (0.2 M). Then, 50 μL of pAb was added to the above solution and left to react for 30 min at room temperature, 40 μL of 10% BSA (w/v) solution was added to block the unbound sites GNPs at room temperature. After 30 min, the mixture was centrifuged at 10000 rpm for 20 min. Finally, the precipitate was resuspended in 200 μL of Tris-HCl solution (0.02 M, pH 8.5) containing BSA

(0.5%, w/v), Tween-20 (0.05%, v/v) and NaN_3 (0.01%, w/v), the last solution was defined as immune probe.

2.8 Strip fabrication of strip

The GNPs immunochromatographic strip consists of a sample pad, a NC membrane, an absorbent pad, and a PVC plate (**Figure 3A**). The appropriate concentration of coating antigen (0.25 mg/mL, APD-OVA and goat anti-rabbit IgG antibody (0.4 mg/mL) diluted by the coating buffer (PBS, 0.01 mol/L, pH 7.4) were coated onto the NC membrane to form the test line (T line) and control line (C line) with the dispense platform (XYZ 3060, Biodot, USA) at a jetting rate of 0.8 μL/cm. Then, the NC membrane was dried at 37 ℃ overnight. The sample pad was pretreated with 0.5% sucrose, 0.3% polyvinylpyrrolidone, 0.5% BSA and 0.5% Tween-20 in Tris-HCl (0.02 mol/L, pH 7.4) and dried at 37 ℃ overnight. The sample pad and absorbent pad were cut into 15 and 25 cm respectively. Subsequently, the NC membrane, the sample pad, and the absorbent pad were sequentially pasted on the PVC plate with an overlap of 1-2 mm. Finally, the assembled plate was cut into 3.05 mm wide strips and kept in a desiccator at room temperature.

2.9 Detection procedure of strip

The GNPs immunochromatographic strip was based on competition for the antibody binding sites among free acetophenetidin and fixed coating antigens sprayed on NC membranes **(Figure 3)**. For the herbal tea samples detection, the samples were diluted with an equal volume of PBS (0.01 M, pH 7.4) to obtain the test solution. Then, a total of 150 μL of this solution was added to a microplate well, 10 μL of GNPs-Abs conjugates were added and mixed incubated for 3 min at room temperature. After incubation, the prepared test strips were vertically inserted into the micropores for

another 5 min chromatographic reaction, and the sample pad was rejected immediately. For qualitative detection, the results of the immunochromatographic strip can be directly observed by naked eye. For quantitative analysis, the test strips were scanned by a reader and the color intensity of T line (T value) was calculated.

2.10 Strip performance

Sensitivity. The sensitivity of *GNPs immunochromatographic strip* was evaluated by analyzing a series of concentrations of acetophenetidin in herbal tea samples. For qualitative detection, the result was obtained by naked eye, cut-off value of the immunochromatographic strip was defined as the concentration to result in no red line on the T line (Yao, et al., 2021). For quantitative detection, the color intensity of T line (T value) on test strips was calculated by the test strips reader and the calibration curve fitted by OriginPro 9.1 software (OriginLab Corp., Northampton, MA, USA) was obtained by plotting T value against the concentration of acetophenetidin. The quantitative limit of detection (qLOD) was defined as the concentration of 10% inhibition (Zhongxing Wang, et al., 2020; Yao, et al., 2021), and the detection range was calculated as the concentration from 20% to 80% inhibition (Zhongxing Wang, et al., 2020; Y. Zhang, et al., 2017).

Specificity. The specificity of the immunochromatographic strip was evaluated by function or structure related drugs, which were ~~possibly~~ added to herbal tea, such as acetaminophen, chlorpheniramine, aminopyrine, metronidazole, phenylbutazone, piroxicam, ibuprofen, diclofenac sodium as the interfering compounds. The interfering compounds were utilized with the concentration at cut-off value for specificity experimental (H. Zhang, et al., 2020). All experiments were carried out three times.

Accuracy and precision of strip. Accuracy and precision of the GNPs immunochromatographic strip were expressed as recovery and as coefficient of

variation (CV), respectively. To evaluate accuracy and precision, the blanked herbal teas were spiked with acetophenetidin at 5.0, 15.0, 45.0 ng/mL.

2.11 Application on real samples

The commercial samples (Collected from herbal tea store at Tianhe District Guangdong Province and some provided by Guangdong Food Inspection Institute, n=20) were analyzed by GNPs immunochromatographic strip and, in addition, by LC-MS/MS, which was performed according to the BJS 201713 developed and published by State Administration for Market Regulation (China). See Supplementary file (State Administration for Market Regulation of China, 2017).

3. Results and discussion

3.1 Hapten design

To produce a sensitive and specific antibody to small molecules, the rational hapten design is usually the most important step (Mi, et al., 2019). The molecular weight of acetophenetidin is only 179.2, which is not immunogenic and is unable to cause an immune response to the animals. Therefore, acetophenetidin need to be coupled with a carrier protein to enhance immunogenicity. Firstly, the acetophenetidin may be considered as a hapten, however, acetophenetidin did not possess a suitable group to couple with the carrier protein. Moreover, it is also difficult to modify the structure of acetophenetidin to introduce another active group. Therefore, acetaminophen (**Figure 1**), a structural analogue of acetophenetidin, was selected as a hapten, because it was more advantageous to modify its structure by replacing the ethoxy group of acetophenetidin with a hydroxyl group at the edge (Han, et al., 2016). To obtain a high performance of antibody-antigen combination, four kinds of spacer

arms were chosen to react with acetaminophen to obtain haptens with carboxyl groups for coupling with carrier proteins. The spacer arms of hapten 1 and 3 were linear alkanes with different lengths, the spacer arm of hapten 4 was an alkane with side chain, and the spacer arm of hapten 2 was aromatic hydrocarbons.

3.2 Characterization of antibody

The ELISA was used to evaluate the performance of the antibody 1 (against hapten-1-BSA) and antibody 2 (against hapten-2-BSA). Under the homologous combination, antibody 1 combined with coating antigen 1 (hapten-1-OVA) showing a high titer, but no significant inhibition was observed even at 1 μg/mL of acetophenetidin and acetaminophen. Similarly, the combination of antibody 2 and antigen 2 showed the same results, high titer, no inhibition (**Table 1**). Comparing with homologous strategy, the heterogeneous strategy could significantly improve sensitivity (Xu, et al., 2010). Therefore, the heterogeneous strategy was adopted to screen suitable coating antigen. When using the coating antigen containing straight chain alkane or aromatic hydrocarbon spacer arms, antibody 1 and antibody 2 both showed weak? sensitivity to acetophenetidin and acetaminophen (**Table 1**). However, antibody 1 and antibody 2 showed better inhibition rate to acetophenetidin and acetaminophen with the coating antigen containing sidebar chain alkane spacer arm (antigen 4, Hapten 4-OVA). Then, the calibration curves were obtained by analyzing a series of standard concentrations of acetaminophen and acetophenetidin (**Figure S1**). The IC_{50} values of acetophenetidin and acetaminophen was 16.46 and 71.21 ng/mL, respectively. Due to its better sensitivity, antibody 1 was selected for the further cross-reactivity investigation (Schelstraete, et al.). The CR by calculating the ratio of IC_{50} for acetophenetidin to other illegally added drugs was evaluated (**Table 2**). The highest CR was observed for acetaminophen (CR = 3.25%), and other drugs showed less than 0.01% CR to the

antibody 1. In this way, antibody 1 showed good sensitivity and specificity that indicated that antibody 1 could be used for the development of an immunochromatographic strip for the detection of acetophenetidin.

3.3 Conformation Analysis

To elucidate the recognition mechanism of antibody with acetophenetidin, acetaminophen and coating antigens, the lowest energy conformational optimizations and the electrostatic potential surfaces of acetaminophen, acetophenetidin and haptens (**Figure 2**) were calculated by molecular modeling. It was found that hapten 4 and hapten 1, 2, 3 exhibit completely different conformations? (**Figure 2**), this was due to the introduction of side chain alkane spacers that changed the conformation of molecules which reduced the affinity of the antibody with the coating antigen. This may be why only hapten 4-OVA was used as the coating antigen, and the antibodies showed specificity for acetophenetidin and acetaminophen. On the other hand, we found that acetophenetidin exhibited completely opposite electrostatic potential with acetaminophen and haptens (**Figure 2**), and the antibodies showed better sensitivity for acetophenetidin than acetaminophen. This may be due to the different electrostatic potential of acetophenetidin and immunogen, which enhanced the affinity of acetophenetidin and antibody, therefore increasing the sensitivity.

3.4 Development of GNPs immunochromatographic strip

After characterizing and selecting the best performed combination of antibody and antigen, the GNPs immunochromatographic strip for detection of acetophenetidin was developed. The principle of GNPs immunochromatographic strip was shown in **Figure 3**. Briefly, acetophenetidin and coating antigen compete for the binding site of antibodies labeled with GNPs. When there was no acetophenetidin present in the

samples, the GNPs-Ab conjugates bound to the antigen coated on the T Line to form a red band. When there was acetophenetidin present in the samples, acetophenetidin bound to the GNPs-Ab conjugates to form a GNPs-Ab-APD complex in the well, which causes the color of T line to weaken until it gradually decreased as the acetophenetidin concentration increases.

In the immunoassay, an adequate condition for detection of a target is indispensable to obtain excellent performance with the pH value, the concentration of antibody and coating antigen and the volume of the immune probe being the key parameters. Hence, several conditions were optimized (**Figure 4**). The pH values of the GNPs solution were optimized by adding different volumes of 0.2 M K_2CO_3 (6, 9, 12, 15, 18, 21 μL) to the tube containing 1 mL GNP solution. Comparing the results of the positive test groups (100 ng/mL acetophenetidin in PBS) and negative test groups (PBS, 0.01 M, pH 7.4), the optimal condition was selected. With regards to K_2CO_3, 9 μL was chosen because the color intensity of T line in negative test groups increased and then decreased with the volume of K_2CO_3 increase, showing the strongest color intensity at 9 μL and a light red band on the T line in positive test groups (**Figure 4A**). Similarly, the concentration of antibody and antigen was optimized with a range of concentrations. The optical concentrations of antibody and antigen were 0.2 mg/mL and 0.06 mg/mL (**Figure 4B, 4C**), respectively. For the volume of immune probe mixed with samples in the microtube, different volume of immune probe (6, 8, 10, 12, 14, 16 μL) was added to optimized and the blank herbal tea and the herbal tea spiked 160 ng/mL acetophenetidin was regarded as negative test groups and positive test groups. Considering the color intensity of the T line in negative test groups and the inhibitory effect in positive test groups, 12 μL of immune probe was chosen for the next experiments.

3.5 Strip performance

3.5.1 Sensitivity

For the sensitivity, a range of concentrations of herbal samples from 0 to 320 ng/mL were analyzed. As expected, the color intensity of T Line gradually weakened and disappeared with the concentration of acetophenetidin increased (**Figure 5**). The cut-off value of the GNPs immunochromatographic strip, defined as the concentration of acetophenetidin resulting in absence of red bands on the T line confirmed by naked eye observation (Yao, 2021 #9), was 160 ng/mL in herbal tea samples. Furthermore, the color intensity of T line (T value) of these spiked acetophenetidin herbal tea with different concentrations were supplied by the reader. Then, the calibration curve fitted by OriginPro 9.1 software (OriginLab Corp., Northampton, MA, USA) was obtained by plotting value against the concentration of acetophenetidin in herbal tea. The qLOD, defined as the 10 % inhibition concentration (IC_{10}) (Guan, et al., 2022), was 1.63 ng/mL and the detection range defined as from 20% inhibition concentration to 80% inhibition concentration was from 3.84 to 72.22 ng/mL. Comparing with Gabriela's portable colorimetric paper-based device for detection of acetophenetidin with LOD of 3500 ng/mL (da Silva, de Araujo, & Paixão, 2018), the GNPs immunochromatographic strip show better sensitivity which is increased by more than 20 times. At the same time, He et al. investigated the contamination of the illegal additives in 456 batches of herbal tea on the market. Among them, 3 batches of herbal beverages were found acetophenetidin with concentration ranging from 49 to 520 mg/mL (He, et al., 2018). Therefore, the GNPs immunochromatographic strip can meet the detection requirements of actual

commercial samples.

3.5.2 Specificity

To investigate the specificity of the GNPs immunochromatographic strip, other drugs illegally added to herbal tea such as acetaminophen, chlorpheniramine, aminopyrine, metronidazole, phenylbutazone, piroxicam, ibuprofen, diclofenac sodium were conducted. The blank herbal tea samples and spiked with drugs with herbal tea samples were detected (**Figure 6**). The color intensity of T line disappeared when the target acetophenetidin existed, while the presence of other interfering compounds did not cause evident changes to the T line or weaken a little bit, and the result was same as result of ELISA. This indicated a good spcificity of the GNPs immunochromatographic strip for acetophenetidin.

3.5.3 Accuracy and precision

For accuracy and precision, blank herbal teas were spiked acetophenetidin with the concentration of 5, 15, 45 ng/mL. The result was listed in Table 3, the recoveries were at a range of 102.2-106.1% and CV below 7.2%. These results indicated that the substance of the real sample did not affect the detection of acetophenetidin in the GNPs immunochromatographic strip. The immunochromatographic strip, therefore, successfully measured the acetophenetidin in herbal tea samples.

3.6 Strip application to real herbal samples

To evaluate the suitability of GNPs immunochromatographic strip for screening acetophenetidin in herbal tea samples, the developed GNPs immunochromatographic strip was used to detect acetophenetidin in 20 real samples collected from herbal tea store at Tianhe District Guangdong Province and provided by Guangdong Food

Inspection Institute that were also applied to LC-MS/MS (**Table 4**). Three positive samples illegally added high-concentration acetophenetidin were found (No. 8, 10 and 13). In addition, other samples were negative samples. Except these samples, other samples were negative samples. Both detection methods show a high degree of agreement, proving that the developed GNPs immunochromatographic strip for detecting acetophenetidin in herbal tea samples has high suitability.

4. Conclusion

In this study, four haptens were designed to prepared for two immunogens and four coating antigens. Two antibodies were firstly generated, and evaluated by ELISA to obtain ultra-sensitivity to acetophenetidin with different coating antigens. IC_{50} was calculated to 16.46 ng/mL for acetophenetidin. CR with acetaminophen, analgin, ibuprofen and other drugs were below 3.5%. Molecular modeling revealed that the immunogen designed with opposite electrostatic potential surfaces to the analyte was conducive to generate highly sensitive antibody. The obtained antibody and coating antigen were used to develop a GNPs immunochromatographic strip for the detection of acetophenetidin in herbal tea, showing a cut-off value of 160 ng/mL by naked eye observation and 1.63 ng/mL by a reader. The standard drugs (aminopyrine, chlorpheniramine, aminopyrine, metronidazole, phenylbutazone, salicylic acid, piroxicam, ibuprofen, diclofenac sodium, dexamethasone, dexamethasone acetate) used to verify the specificity, and the CR of the new GNPs immunosensor were all below 4%. The accuracy and precision were evaluated by analysis of spiked herbal samples resulting in recoveries of 102.2% to 106.1% and coefficients of variation below 8%. In addition, the reliability of the immunosensor was confirmed by the LC–MS/MS method showing high consistency. All the above show that the proposed strip assay was

proved to be ideal for an on-site screening surveillance purpose for acetophenetidin in herbal tea, and the hapten strategy followed in this work could be used for similar compound antibody preparation in the future.

Abbreviations:

acetophenetidin, APD; High-performance liquid chromatography, HPLC; High-performance liquid chromatography-tandem mass spectrometry, HPLC-MS/MS; Ultra-performance liquid chromatography-tandem mass spectrometry, UHPLC-MS/MS; Gas chromatography mass spectrometry, GC-MS; Bovine serum albumin, BSA; Ovalbumin, OVA; Enzyme-linked immunosorbent assay, ELISA; 3,3',5,5'-tetramethylbenzidine, TMB; N-hydroxysuccinimide, NHS, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EDC; N, N-Dimethylformamide, DMF; Acetaminophen, ACE; Antibody, Ab; Antigen, Ag; half maximal inhibitory, IC_{50}; gold nanoparticles, GNPs; Cross-reactivity, CR quantitative limit of detection, qLOD; Coefficient of variation, CV.

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References

da Silva, G. O., de Araujo, W. R., & Paixão, T. R. (2018). Portable and low-cost colorimetric office paper-based device for phenacetin detection in seized cocaine samples. *Talanta, 176*, 674-678.

Dubach, U. C., Rosner, B., & Stürmer, T. (1991). An epidemiologic study of abuse of analgesic drugs: effects of phenacetin and salicylate on mortality and cardiovascular morbidity (1968 to 1987). *New England Journal of Medicine, 324*(3), 155-160.

Fiorentin, T. R., Logan, B. K., Martin, D. M., Browne, T., & Rieders, E. F. (2020). Assessment of a portable quadrupole-based gas chromatography mass spectrometry for seized drug analysis. *Forensic Science International, 313*, 110342.

Ghasempour, H., Dehestani, M., & Hosseini, S. M. A. (2020). Theoretical studies of the paracetamol and phenacetin adsorption on single-wall boron-nitride nanotubes: a DFT and MD investigation. *Structural Chemistry, 31*(4), 1403-1417.

Guan, T., Shen, Y., Jiang, Z., Zhao, Y., Liang, Z., Liu, Y., Shen, X., Li, X., Xu, Z., & Lei, H. (2022). An ultrasensitive microfluidic chip-based immunoassay for multiplex determination of 11 PDE-5 inhibitors in adulterated health foods. *Sensors and Actuators B: Chemical*, 131450.

Han, W., Pan, Y., Wang, Y., Chen, D., Liu, Z., Zhou, Q., Feng, L., Peng, D., & Yuan, Z. (2016). Development of a monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay for nitroimidazoles in edible animal tissues and feeds. *Journal of pharmaceutical and biomedical analysis, 120*, 84-91.

He, J., Wen, J., Lai, Y., & Cao, Y. (2018). Determination of 28 exogenous medicines and endogenous components in herbal drink using high performance liquid

chromatography. *Se pu= Chinese Journal of Chromatography, 36*(8), 758-765.

Kankuri, E., Solatunturi, E., & Vapaatalo, H. (2003). Effects of phenacetin and its metabolite p-phenetidine on COX-1 and COX-2 activities and expression in vitro. *Thrombosis research, 110*(5-6), 299-303.

Landwehr, K. R., Larcombe, A. N., Reid, A., & Mullins, B. J. (2021). Critical Review of Diesel Exhaust Exposure Health Impact Research Relevant to Occupational Settings: Are We Controlling the Wrong Pollutants? *Exposure and Health, 13*(2), 141-171.

Li, X., Wu, X., Wang, J., Hua, Q., Wu, J., Shen, X., Sun, Y., & Lei, H. (2019). Three lateral flow immunochromatographic assays based on different nanoparticle probes for on-site detection of tylosin and tilmicosin in milk and pork. *Sensors and Actuators B: Chemical, 301*, 127059.

Mi, J., Dong, X., Zhang, X., Li, C., Wang, J., Mujtaba, M. G., Zhang, S., Wen, K., Yu, X., & Wang, Z. (2019). Novel hapten design, antibody recognition mechanism study, and a highly sensitive immunoassay for diethylstilbestrol in shrimp. *Analytical and bioanalytical chemistry, 411*(20), 5255-5265.

Salles, M. O., Araujo, W. R., & Paixão, T. R. (2016). Development of a molecularly imprinted modified electrode to evaluate phenacetin based on the preconcentration of acetaminophen. *Journal of the Brazilian Chemical Society, 27*, 54-61.

Schelstraete, W., Devreese, M., & Croubels, S. (2018). Storage stability study of porcine hepatic and intestinal cytochrome P450 isoenzymes by use of a newly developed and fully validated highly sensitive HPLC-MS/MS method. *Analytical and bioanalytical chemistry, 410*(6), 1833-1843.

Wang, J., Shen, X., Zhong, P., Li, Z., Tang, Q., Huang, X., Zherdev, A. V., Dzantiev, B. B., Eremin, S. A., & Xiao, Z. (2021). Heterologous immunoassay strategy for

enhancing detection sensitivity of banned dye rhodamine B in fraudulent food. *Chemical and Biological Technologies in Agriculture, 8*(1), 1-10.

Wang, Z., Guo, L., Liu, L., Kuang, H., Xiao, J., & Xu, C. (2020). Development and comparison of two nanomaterial label-based lateral flow immunoassays for the detection of five antibacterial synergists. *New Journal of Chemistry, 44*(38), 16501-16510.

Wang, Z., Sun, W., Lin, Z.-F., Sun, R., Huang, C.-K., Ye, W.-J., Dong, Y.-Y., Zhang, X.-D., & Chen, R.-J. (2019). A UHPLC-MS/MS method coupled with liquid-liquid extraction for the quantitation of phenacetin, omeprazole, metoprolol, midazolam and their metabolites in rat plasma and its application to the study of four CYP450 activities. *Journal of pharmaceutical and biomedical analysis, 163*, 204-210.

Xie, H., Li, Y., Wang, J., Lei, Y., Koidis, A., Li, X., Shen, X., Xu, Z., & Lei, H. (2022). Broad-specific immunochromatography for simultaneous detection of various sulfonylureas in adulterated multi-herbal tea. *Food chemistry, 370*, 131055.

Xu, Z.-L., Shen, Y.-D., Zheng, W.-X., Beier, R. C., Xie, G.-M., Dong, J.-X., Yang, J.-Y., Wang, H., Lei, H.-T., & She, Z.-G. (2010). Broad-specificity immunoassay for O, O-diethyl organophosphorus pesticides: application of molecular modeling to improve assay sensitivity and study antibody recognition. *Analytical chemistry, 82*(22), 9314-9321.

Yao, J., Wang, Z., Guo, L., Xu, X., Liu, L., Kuang, H., & Xu, C. (2021). Lateral flow immunoassay for the simultaneous detection of fipronil and its metabolites in food samples. *Food Chemistry, 356*, 129710.

Yin, H., Meng, X., Xu, Z., Chen, L., & Ai, S. (2012). Electrochemical behavior of phenacetin on CdSe microspheres modified glassy carbon electrode and its simultaneous determination with paracetamol and 4-aminophenol. *Analytical*

Methods, 4(5), 1445-1451.

Zhang, H., Wang, L., Yao, X., Wang, Z., Dou, L., Su, L., Zhao, M., Sun, J., Zhang, D., & Wang, J. (2020). Developing a simple immunochromatography assay for clenbuterol with sensitivity by one-step staining. *Journal of agricultural and food chemistry, 68*(52), 15509-15515.

Zhang, Y., Wang, L., Shen, X., Wei, X., Huang, X., Liu, Y., Sun, X., Wang, Z., Sun, Y., & Xu, Z. (2017). Broad-specificity immunoassay for simultaneous detection of ochratoxins A, B, and C in millet and maize. *Journal of agricultural and food chemistry, 65*(23), 4830-4838.

Zhao, J., Lv, G.-P., Chen, Y.-W., & Li, S.-P. (2011). Advanced development in analysis of phytochemicals from medicine and food dual purposes plants used in China. *Journal of Chromatography A, 1218*(42), 7453-7475.

Zou, X., Chen, C., Huang, X., Chen, X., Wang, L., & Xiong, Y. (2016). Phage-free peptide ELISA for ochratoxin A detection based on biotinylated mimotope as a competing antigen. *Talanta, 146*, 394-400.

Figure captions

Figure 1. The synthetic scheme for acetophenetidin hapten

Figure 2. The scheme of GNPs immunochromatographic strip. (A) Labeling antibody with GNPs. (B) Structure of the immunochromatographic strip. (C) Test procedure of immunochromatographic strip.

Figure 3. Lowest energy conformations and molecular electrostatic potential isosurfaces of acetophenetidin (A), acetaminophen (B) and haptens (E, F, H, G). Electrostatic potential (EP) was from crimson (highest positive electrostatic) to purple (lowest negative electrostatic).

Figure 4. The optimization for GNPs immunochromatographic strip. (A) The volume of 0.2 M K_2CO_3 (6, 9, 12, 15, 18, 21). (B) The concentration of antibody (0.05, 0.1, 0.2, 0.25, 0.4, 0.35 mg/mL). (C) The concentration of antigen (0.04, 0.06, 0.08, 0.10 ng/mL). (D) The volume of immune probe (6, 8, 10, 12, 14 μL).

Figure 5. The sensitivity of GNPs immunochromatographic strip for detection of acetophenetidin in herbal tea by naked eyes and a reader.

Figure 6. The specificity research results of the GNPs immunochromatographic strip for acetophenetidin detection. The concentrations of all evaluated analytes in herbal tea are 160 ng/mL