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Using a quantum dot bead-based lateral flow immunoassay to broadly detect the adulteration of PDE-5 inhibitors in functional foods

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Abstract

In this study, a designed hapten possessing a classic structure of PDE-5 inhibitors was synthesized. A monoclonal antibody (mAb) with broad recognition ability of six PDE-5 inhibitors was further induced. For the determination of lodenafil, methisosildenafil, mirodenafil, udenafil and tadalafil, the limit of detection (LOD) and IC50 ranged from 1.01 to 26.91 ng/mL, and 12.75 – 278 ng/mL, respectively. Thereafter, a quantum dot beads based lateral flow immunoassay (QBs-LFIA) was developed, which promoted the LOD and IC50 to 0.32– 6.52 ng/mL, and 7.45–133.8 ng/mL, respectively. Method validation was conducted using honey, capsule samples spiked with PDE-5 inhibitors, and the recoveries of intra and inter assay ranged from 81.01% to 108.16%, with coefficients of variation below 12.71%. In addition, the validity and the consistency has been confirmed with the comparison between QBs-LFIA and HPLC-MS/MS (R^2 12 = 0.9957).

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Author contributions

Conceptualization, C. Z.; methodology, Q. W.; soft - ware, M. S. and B.L.; validation, M. S., Q. W. and P.L.; formal analysis, M. S.; resources, Q.W., S. D. and L.J.; writing – original draft preparation, Q. W.; writing – review and editing, C. Z. B. D. H.; supervision, Y. X., and C. Z.; funding acquisition, C. Z.; All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Ethics Statements

This study was performed with the approval of Animal Welfare Committee (AWC) of Jiangsu Academy of Agricultural Sciences, and in compliance with the Regulations for the administration of affairs concerning experimental animals, P.R. China. All the above mentioned guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Furthermore, the developed QBs-LFIA was employed for real products inspection, several samples was found to be adulterated by lodenafil, methisosildenafil.

Keywords

Broad recognition of monoclonal antibody; PDE-5 inhibitors; Quantum dot beads; Lateral flow immunoassay

Introduction

The demands for functional food and health-promoting products, particularly natural or herbal products, are projected to increase in China[1]. The rapidly increasing aging population specifically benefits from functional foods because of age-related health issues and disease threats [2,3]. For example, ingredients of isoflavones, saponins, phytic acids, phytosterols, and soy-based peptides have shown use in cholesterol reduction, cardiovascular disease prevention, diabetic symptoms prevention, and so on [4]. However, the illegal addition of prohibited chemicals in functional food or adulterated health-promoting products has raised public concern. For instance, phosphodiesterase-5 31 (PDE-5) inhibitors, such as sildenafil, mirodenafil, tadalafil, and udenafil, are prohibited drugs but frequently found in some herbal health-promoting products, which could pose threats to consumers' health[5,6]. Moreover, many analogs of PDE-5 inhibitors have also been discovered in adulterated functional food and health products[7–11]. The illegal addition of PDE-5 inhibitors and their analogs is aimed to give the 39 customers false pleasant feeling, mislead the effects of 40 functional food and promote further comsumption. It could put the consumers, especially the middle-aged and elderly, at great risk of physical aches, pains, and death[12]. In this regard, it is necessary to develop reliable detection methods of PDE-5 inhibitors to prevent adulterations in functional foods and health-promoting products. A simple, fast, and accurate analytical method for on-site detection would be ideal.

Instrumental methods, such as liquid chromatography-mass spectrometry (LC/MS) and gas chromatography-mass spectrometry (GC/MS)[13,14], have the advantage of high sensitivity and specificity for the quantitative detection of PDE-5 inhibitors. However, such methods require trained operators and intricate equipment, which limits their use in rapid on-site detection. Meanwhile, rapid detection methods could provide preliminary screening results to assist quantitative detection.

Immunological analysis based on specific recognition between antigen and antibody has been widely applied in food safety and environmental monitors, such as enzyme-linked immunoassay (ELISA)[15–17] and lateral flow immunoassay (LFIA)[18–20]. LFIA combined with gold nanoparticles has been developed to meet the on-site detection requirement with lower cost in many applications [21–23]. Meanwhile, fluorescent systems with signal amplification and magnetic nano-materials with strong enrichment ability have been introduced in LFIA to enhance the sensitivity[24–26]. Quantum dots are known for their broad excitation spectra, narrow emission spectra, and large molar attenuation coefficient[27,28]. Therefore, quantum dot nanobeads (QBs) where QDs were embedded can be used as an

outstanding fluorescence-labeling material to improve the detection sensitivity of LFIA[29,30]. QBs-based LFIA is an ideal method for the sensitive detection of PDE-5 inhibitors.

The antibodies play a key role in the detection of PDE-5 inhibitors[31]. Song et al. reported that a carboxyl group was introduced to the sildenafil to form the hapten, and the resulting polyclonal antibody recognized sildenafil, vardenafil, and tadalafil with half-maximal inhibitory concentration (IC_{50}) of 6, 60 and 250 ng/mL, respectively[32]. He et al. synthesized a hapten by modifying the tadalafil structure, and the induced polyclonal antibody showed broad recognition against tadalafil and its analogs (amino tadalafil, acetami-notadalafil, nortadalafil) with IC_{50} of 0.90, 0.88, 1.06, and 1.33 ng/mL[33]. The above-mentioned polyclonal antibodies could broadly recognize PDE-5 inhibitors by targeting different epitopes, but there was a lack of stability due to the mixed subtypes[34]. Guo et al. produced a monoclonal antibody (mAb) using vardenafil analogs as hapten, and the obtained antibody could recognize vardenafil with IC_{50} of 18.2 ng/mL[35]. Wang et al. also produced a mAb targeting sildenafil with an IC_{50} of 0.52 ng/mL[36].

However, a number of PDE-5 inhibitor derivatives with variable structures have been derived from the first-generation inhibitors led by sildenafil to form a series of second-generation inhibitors led by tadalafil and mirodenafil[37]. The second generation inhibitors bring new challenges for the adulteration testing. A broad recognition method is necessary to provide fast on-site screening of PDE-5 inhibitors.

In this study, we designed a generic hapten in order to produce the mAb with broad recognition for PDE-5 inhibitors. Furthermore, we aimed to develop QBs-LFIA to meet the need of rapid and simultaneous screening of PDE-5 inhibitors in functional food.

Materials and methods

Reagents and apparatus

N-(2-Hydroxyethyl)piperazine,N-(3(Dimethylamino)propyl)-N’-ethylcarbodiimide(EDC·HCl), dicyclohexylcarbodiimide (DCC), nhydroxysuccinimide (NHS) and succinic anhydride were purchased from Aladdin (Shanghai, 117 China). Sildenafil (SID), hydroxyhomo sildenafil (lodenafil, LD), acetildenafil (ACE), tadalafil (TARD), mirodenafil (MID), udenafil (UD), and methisosildenafil (ME-SID) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Ovalbumin (OVA), bovine serum albumin (BSA), polyethylene glycol 2000, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine thymidine (HT) medium, peroxidase-labeled goat anti-mouse IgG, tetramethylbenzidine (TMB) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Balb/c mice were purchased from Yangzhou UniversityMedical Comparison Center (Yangzhou, Jiangsu). Nitrocellulose (NC), sample pad, membrane, and absorbent pad were provided by Schleicher and Schuell GmbH (Dassel, Germany), Phosphate buffered saline (PBS, 0.01 M, with 137 mM NaCl, pH = 7.4) and carbonate buffer (with 15 mM Na2CO3 and 35 mM 135 NaHCO3, pH = 9.6) was obtained from Sangon biotech (Shanghai, China). QBs (λem = 625 nm) where CdSe/ZnS QDs embedded in were provided by State Key Laboratory of Food Science and Technology, Nanchang University.

The products of real samples in the type of tablet, capsule and oral liquids were purchased online from e-commerce platforms. All of the other reagents were of analytical grade.

Cell culture plates and 96-well polystyrene microplates were purchased from Costar (Corning, NY, 145 USA). The CO_2 incubator and ELISA absorbance reader were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The XYZ 3050 dispensing platform and CM4000 Guillotine Cutter (BioDot, Irvine, CA) were used to prepare the test trip. The fluorescence intensity of Test line and Control line on the QBs-LFIA was measured by a fluorescent strip reader (FIC-S2011-L44) with λex=365 nm and λem=625 nm, which was purchased from Suzhou Hemai Percision Instrument Co., Ltd. (Jiangsu, China). The photos of QBs-LFIA were taken under UV-based analyzer ZF-1 from Lichen Co., Ltd. 138 (Shanghai, China).

Synthesis of hapten and antigen

As shown in Fig. 1, compound 1 (N-(2-Hydroxyethyl)piperazine, 13 mg, 0.1 mmol) and compound 2 (3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1H-pyrazolo[4,3-d]pyrimidin-5-yl)benzenesulfonyl chloride, 36.6 mg, 0.1 mmol) were reacted in tetrahydrofuran at room temperature (RT) for 6 h. The solution was extracted with ethyl acetate, which was removed at 60 °C for 10 h to obtain compound 3. Then, compound 3 (92 mg, 0.2 mmol) was dissolved in 15 mL of DMF and added with succinic anhydride (20 mg, 0.02 172 mmol), stirred overnight at RT. The product was extracted with ethyl acetate, and the obtained yellow residue of hapten ((2-(4-(4-ethoxy-3-(1-methyl-7-oxo-3propyl-6,7-dihydro1H-pyrazolo[4,3-d]pyrimidin-5yl)phe-nylsulfonyl)piperazin-1-yl) acetic acid) was dried at 60 °C for 3 h.

The antigen (hapten-BSA) was prepared as an immunogen using the active ester method. In brief, 15.7 mg of hapten (0.025 mmol), 20.5 mg of EDC (1.0 mmol), and 28.75 mg of NHS (0.25 mmol) were dissolved in 1.5 mL of DMSO. The mixture was stirred overnight at RT. Then, 41.25 mg of BSA (0.001 mmol) diluted with 5 mL of carbonated buffer was placed in the activated solution for 2 h reaction at RT. The product was dialyzed with PBS at 4 °C and the fluid was changed every 8 hours for 3 days. The final product was stored at −20 °C for further use. The coating antigen (hapten-OVA) was synthesized with the same methods, and the feeding molar ratio between hapten and OVA was 15:1.

Production of the mAb against PDE-5 inhibitors

Following the methods described in the previous work[38], five BALB/c mice at 6 weeks were injected subcutaneously with the immunogen (hapten-BSA). In brief, 100 μg of immunogen (dissolved in PBS) was mixed with an equal volume of Freund's complete adjuvant for the first immunization. The adjuvant was replaced with an incomplete one in the following steps, and immunization was implemented every 3 weeks. The titer and target recognition of serum were assessed by ELISA. Eventually, the mice was sacrificed for spleen cell collection and further fusion with myeloma cells (SP2/0). All experiments were performed in compliance with the relevant laws and institutional guidelines (Regulations for the administration of affairs concerning experimental animals, P.R. China), and Animal

Welfare Committee (AWC) of Jiangsu Academy of Agricultural Sciences have approved the experiments.

Hybridoma technology was used in cell fusion according to the previous work[39], and positive hybridoma cell clones were selected and characterized by ic-ELISA after 7–10 days. A specific monoclonal cell was obtained from a positive hybridoma cell line after 4 times of subcloning using the same procedure. IC_{50} was defined as the concentration with 50% inhibition of maximal absorbance. The limit of detection (LOD) was equivalent to the IC_{10} value, which was defined as the 10% of the maximal absorbent concentration of PDE-5 inhibitors. The ascites was prepared by injecting postpartum mice with screened hybridoma cells and stored at −20 °C for further use.

Procedure of ic-ELISA

The ELISA plates were coated with 100 μL of coating antigen which was diluted to 1 μg/mL with carbonate buffer and incubated at 4 °C for 12 h. The plates were washed with PBST (2 L PBS with 1 mL Tween-20) for 3 times. Then 200 μL blocking buffer (PBS with 3% skim 230 milk) was added to the wells for 2 h at 37 °C and the plates were washed for 3 times. 50 μL of PDE-5 standard solutions or sample extracts were added to the wells, respectively, and 50 μL of diluted antibody solution was added to the wells too. After an incubation of 1 h at 37 235 °C, the plates were washed for another 3 times. 100 μL Goat anti-mouse IgG (HRP labeled) (1:5000) was added and incubated at 37 °C for 1 h. After washing for 5 times,100 μL TMB substrate was added and incubated for 15 min at 37 °C. Finally, 50 μL of H2SO4 (2 M) was used to stop the enzymatic reaction, and the OD values at 450 nm were measured by the reader.

Characterization of mAb

Antibody quality plays an essential role in target recognition. Affinity and specificity were important factors for the development of a highly sensitive QBs-LFIA. Cross-reactivity (CR) was used to evaluate the nature of mAb (broad-spectrum), which was calculated by (IC_{50} of Lodenafil)/ (IC_{50} of analogs) ×100%, and IC_{50} was calculated from the stand curve.

Preparation of detection probe

The QBs-mAb conjugates were synthesized according to previous work[40]. In brief, 10 μL of QBs (12.5 mg/mL) with a carboxyl group (12.5 mg/mL) was activated with EDC ▫ HCl (1 mg/mL) in 1 mL of phosphate buffer (PB, 0.01 M, pH = 6.0) for 10 min, and 19 μL of the prepared ascites (1 mg/mL) was added in the active solution for 30 min reaction. Then, 10% (w/v) filtered BSA solution was added into the reaction system and stirred for 30 min to block excess nonspecific binding sites and suppress the nonspecific binding. After centrifugation (20,000 × g, 15 min), the detection probe was successfully produced by reconstituting sediment with 1 mL of PBS containing 2% fructose, 1% PEG-20000, 5% sucrose, 1% BSA, and 0.4% Tween-20 for further use.

Preparation of immunochromatographic strips

As shown in Fig. 2, the composition of the lateral flow strip includes a sample pad, NC membrane, and absorbent pad. Hapten-OVA and anti-mouse IgG (1 272 mg/mL) were

sprayed on the NC membrane as test line and control line with the speed of 0.6 μL/cm, using the XYZ-3050 dispensing platform. After drying the NC membrane at 37 °C overnight and assembling it on a polyvinyl chloride (PVC) backing card, the sample pad and absorbent pad were pasted on a PVC card with a distance of 2 mm from the NC membrane. Finally, the assembled strip was cut to 3.5 mm width and stored at a dry environment for further use.

Assay procedure

The developed QBs-LFIA for PDE-5 inhibitors was based on a competition mechanism (Fig. 2). PDE-5 inhibitors and PDE-5 hapten-OVA were combined with the QBs-mAb competitively. With the increase of positive sample, the prepared antigen-mAb-QBs conjugates could not be captured by the T line. The fluorescence signal was measured at 620 nm under excitation of 365 nm. The sample was dissolved in 70 μL of running buffer (5% methanol–PBS solution) and incubated with 2 μL of QBs-mAb probes (40 mg/mL) for 5 min. Then, the incubated solution was added on the sample pad, and the fluorescence intensity at the reaction time of 15 min was recorded. PDE-5 inhibitors standards were dissolved in methanol for long-term storage, further diluted to a series of concentrations with PBS contains 5% methanol for standard curve testing. The standard curve was depicted by B/B_0 where B and B_0 represent the FI_T/FI_C of the sample containing analytes and no target compounds, respectively.

Method validation

The PDE-5 inhibitor-free honey samples or the contents of capsule were spiked for recovery experiment, which was confirmed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [8,41]. The sample extraction was as follows: 1 mL of honey (for capsule, tablet 1.0 g contents) spiked with PDE-5 inhibitors standards was mixed with 2.5 mL of methanol and added 1 g of NaCl, ultra-sonicate for 15mins. After ultra-sonication, the mixture was centrifuged at 4000 g for 15 min, and standing a while for separation, supernatant was collected and filtered with a 0.22 μm filter and evaporated to dryness under nitrogen steam. The extract was redissolved in 2.5 mL of running buffer (PBS contains 5% methanol). Upon testing, the extracts were diluted 50 times using running buffer.

The obtained extract was further analyzed with QBs-LFIA and HPLC-MS/MS for comparison. For QBs-LFIA, the intra-assays were performed for five replicate tests, whereas the inter-assays were analyzed once a day for 3 sequential days. HPLC-MS/MS was used to verify the reliability of QBs-LFIA for PDE-5 inhibitor testing according to the national standard of China (BJS 201805) and studies reported[42,43]. The condition of HPLC-MS/MS system was performed as follows: a) Chromatographic column: Agilent-6410 SB-18 column (3.5 μm, 2.1 × 100 mm inner diameter); b) mobile phase: 20% solvent A (0.1% formic acid in water, v/v) and 80% solvent B (acetonitrile in water, v/v); c) flow rate: 0.2 mL/min at RT; d) Column temperature: 38 °C; e) Ion source: electrospray ionization source (ESI); f) Scan mode: positive ion; g) Detection mode: multiple reaction monitoring (MRM); h) Drying gas: N2 of 11 L/min at 300 °C; i) Monitoring ions pairs (m/z), fragmentor and collision energy.

Results and discussion

Characterization of hapten and antigen

The synthesized hapten was verified by TOF-MS and 1H-NMR. C27H36N6O8S calculated with 604.60 m/z reached 605.24 [M+H]+ by TOF-MS (ESI, Figure S1). The following results from 1H-NMR (300 MHz, CD3OD, Figure S2) showed that the hapten was synthesized successfully with a carboxyl group: δ12.23 (s, 1H), 7.84 (d, 1H), 7.81 (s, 1H), 7.39–7.37 (d, J = 6 Hz, 1H), 4.16 (s, 3H), 4.19–4.24 (m, 2H), 2.88 (s, 4H), 2.79–2.76 (t, J = 5.4 Hz, 2H), 2.50–2.48 (d, J = 8.7 Hz, 2H), 2.37–2.34 (t, J = 4.5 Hz, 2H), 1.79–1.71 (m, 2H), 1.35–1.31 (t, J = 5.1 Hz 3H), 0.96–0.92 (t, J = 5.4 Hz, 3H).

BSA and OVA were conjugated with the carboxyl group of the hapten, which were used as the immunogen and coating antigen, respectively. Based on the SDS-PAGE results (Figure S3(A)), a significant shift was observed, indicating successful conjugation. The conjugation ratio of hapten–BSA was further identified as 25:1 by MALDI-TOF/TOF (Figure S3(B)).

Characterization of mAb

In this study, the hybridoma cell line secreted mAb against PDE-5 inhibitors, which has been identified as IgG1 subclass (Figure S4). Ascites was generated by injecting the appropriate hybridoma cell line into mice. The ascites fluid contained the antibodies for the anti-PDE-5 inhibitors. The specificity of the antibodies was evaluated by performing ic-ELISA cross-reactions against the analogs of PDE-5 inhibitors. As shown in Table S1, the IC_{50} for lodenafil, methisosildenafil, and sildenafil was 12.75, 13.30, and 7.79 ng/mL, whereas the IC_{10} was 1.30, 1.16, and 1.01 ng/mL, and the CR was 100%, 95.8%, 375 and 164%, respectively. For the other PDE-5 inhibitors (mirodenafil, udenafil, tadalafil), IC_{50} was 96, 153, and 278 ng/mL, and IC_{10} was 9.34, 18.13, and 26.91 ng/mL respectively. The CR was below 13.3% for other three PDE-5 inhibitors recognition. The above-mentioned results indicated that the antibody had broad recognition of PDE-5 inhibitors.

QBs-LFIA fabrication and Optimization

As shown in Fig. 2, the QBs-LFIA was assembled and optimized for better performance. First, the coupling efficiency between QBs and mAb was influenced by the pH and concentration of EDC. The pH of the conjugation condition could affect the biological activities of mAb at different isoelectric points and further influence the sensitivity of the QBs-LFIA. The coupling efficiency was measured with FI_T by running the designed QBs probes on the test strip. As shown in Fig. 3(A), the fluorescence intensity increased significantly from 229.7 ± 8.62 to 517.66 ± 40.41 when the pH increased from 5 to 6.5. However, further increasing of the pH from 6.5 to 8 resulted in a sharp decrease of FI_T. Adjusting the concentration of EDC was also important to avoid unwanted conjugations during the carboxyl activation process. As shown in Fig. 3(B), FI_T reached the maximum of 1017 ± 53.03 when the concentration of EDC was 240 406 μg/mg. Therefore, the optimized pH and concentration of EDC were set at 6.5 and 240 μg/mg, respectively, to achieve higher fluorescent response.

Checkerboard strategy ELISA was applied to determine the optimal concentrations of the coating antigen and the QBs-mAb probes, which played a key role in enhancing the sensitivity. The inhibition rate was calculated on the basis of the following equation: (1−B/B0) 414 × 100%, where B and B0 represent FI_T/FI_C of positive and negative samples of PDE-5 inhibitors at 10 ng/mL, respectively. Table 1 shows that the highest (54.16% ± 1.14%) inhibition rate with relatively high FI_T (670 ± 22) was achieved when 2 μL probes were paired with 1 mg/mL hapten-OVA.

The immunoreaction dynamics can also be reflected by the ratio of FI_T/FI_C. A series of spiked solutions with various concentrations of PDE-5 inhibitors (0, 1, 10, and 125 ng/mL) was continually measured and recorded during a 30 min reaction. The results showed an increasing FI_T along with the reaction time. Despite some slight fluctuations, the FI_T/FI_C ratios were stabilized within 15 min, and remained stable thereafter. By considering appropriate fluorescence intensity, the result-reading time was finally set at 15 min.

The pH and methanol content of the running buffer were also evaluated to enhance the reproducibility and sensitivity of the QBs-LFIA. The FI_T and FI_C of negative sample solution and inhibition rates of samples spiked with PDE-5 inhibitors were evaluated in PBS at multiple pHs(5.0, 6.0, 7.0, 7.5, 8.0, and 9.0). The Inhibition rate was defined as (1-B/B0) × 100%, where B_0 and B indicated FI_T/FI_C of the negative and positive sample solution with 10 ng/mL lodenafil. As shown in Fig. 4(B), when the pH was 7.5 and 8.0, the inhibition rates were the highest at 49.62% ± 1.84 % and 51.34% ± 5.79%, respectively. The FI_T and FI_C at pH = 8.0 were drastically lower than that pH = 7.5. Therefore, for real sample testing, the pH of the sample solution should be adjusted to 7.5 to maintain the higher sensitivity of QBs-LFIA. Methanol was used to enhance the solubility of hydrophobic PDE-5 inhibitors. High concentrations of methanol would also affect antibody activity. The methanol content in the solution should be adjusted properly to minimize interference. The inhibition rates of 10 ng/mL lodenafil were evaluated with spiked samples containing a variety of methanol contents (0%, 5%, 10%, 15%, and 20%). Fig. 4(C) shows that 5% methanol content achieved the highest inhibition rate of 55.75% ±1.05%. Under the above-mentioned optimal conditions, a series of lodenafil solutions with different concentrations (three parallels for each concentration, n=3) was tested to construct a standard curve. Fig. 4(D) shows a linear range of 0.39–400 ng/mL and IC_{50} of 9.76 ng/mL for lodenafil. The regression equation of the standard curve was $y = -14.44\ln(x) + 0.8184$ ($R2 = 0.9919$) with IC10 of 0.68 ng/mL. Fig. 4(E) presents the results of the Iodenafil in spiked samples at the concentration of 0, 1, 10, 100, and 200 ng/mL. Compared with negative samples, the fluorescence intensity of the test line decreased along with the increase of lodenafil concentration. The specificity and sensitivity of the test strips for the other PDE-5 inhibitors (sildenafil, methisosildenafil, mirodenafil, udenafil, tadalafil) was evaluated with CR. As shown in Table 2, the CR was 131%, 44.8%, 7.2%, 8.1%, 10.9%, respectively. In addition, the LOD of these five analogs was established at 0.32, 1.09, 2.10, 6.52, and 4.37 ng/mL, respectively (Figure S5).

As shown in table S2, the reported studies on the immunoassay development for the determination of PDE-5 inhibitors were compared with this study. The results showed that the approach developed in this study possessed a broader ability than the other reported assay, and the sensitivity is suitable for the determination of PDE-5 inhibitors.

Matrix effect

The matrix interference could result in false-positive or negative discrimination of testing. In this study, sample dilution was used to reduce the effect of the matrix on the performance of the QBs-LFIA. The result in Fig. 5 indicated that there was interference from the honey matrix that could result in false-positive results. 50-fold dilution could reduce the matrix interference of honey and maintain the sensitive performance of the QBs-LFIA with running buffer. Most of the capsules and tablets are dissoluble in PBS and it leads to negligible matrix effects of capsules and tablets. (Table S3).

Method validation with spiked samples

As shown in Table 3, the recovery of intra-assay and inter-assay was ranged from 70.11%–128.75% and 69.16% - 127.51%, and the CV of intra-assay and inter-assay was 2.49%–14.91% and 1.38%–17.60%, respectively. Meanwhile, the consistency of test strips was verified with HPLC-MS/MS, with LOQ of 0.1–2.5 mg/kg or 0.1–0.5 mg/L[8, 41]. The correlation coefficient was 0.9957 (Fig. 6), indicating the high accuracy of the QBs-LFIA.

Analysis of real samples

Ten commodities in the forms of tablet, capsule, and oral liquid from different manufactures were determined by QBs-LFIA and HPLC-MS/MS for comparison. As shown in Table 4, one of ten tablets and two of ten capsules were found to be positive by QBs-LFIA, which were further characterized by HPLC-MS/MS to be methisosildenafil and lodenafil. All honey samples were confirmed to be negative by both methods. It was noted that the positive tablets and capsules were suspected previously for adulteration of sildenafil and vardenafil then qualified to be negative on HPLC-MS/MS. The QBs-LFIA with broad recognizing ability could provide effective preliminary screening for onsite adulteration testing.

Conclusion

In this study, a broad-recognition mAb was produced based on a designed hapten, which resembled the general structure of PDE-5 inhibitors. A monoclonal antibody was produced and characterized to be able to recognize six kinds of PDE-5 inhibitors, namely, lodenafil, sildenafil, methisosildenafil, mirodenafil, udenafil, and tadalafil. Thereafter, a QBs-LFIA was developed for the determination of PDE-5 inhibitors (lodenafil, sildenafil, methisosildenafl, mirodenafil, udenafil, and tadalafil) with the LOD reached to 0.68, 0.32, 1.09, 2.10, 6.52 and 4.37 ng/mL, respectively. Method validation was conducted with spiked honey and capsule sample, which revealed reliable results and consistency with HPLC-MS/MS. Further verification might be required to characterize its ability for the recognition of other PDE-5 derivatives. The method was tested with real samples and confirmed to be consistent with the results of HPLC-MS/MS. It shall be aware that the derivatives, and analogues of sildenafil was found to be used for adulteration. Large scope inspection for the adulteration of PDE-5 inhibitor is necessary. The QBs-LFIA developed in this study has great potential to be further used as an on-site screening tool for the adulterations of PDE-5 inhibitors in functional food and drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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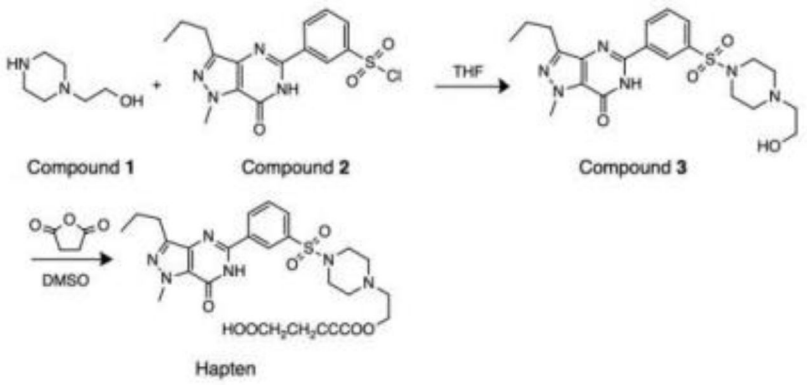


Fig. 1.
Synthetic route of the hapten.

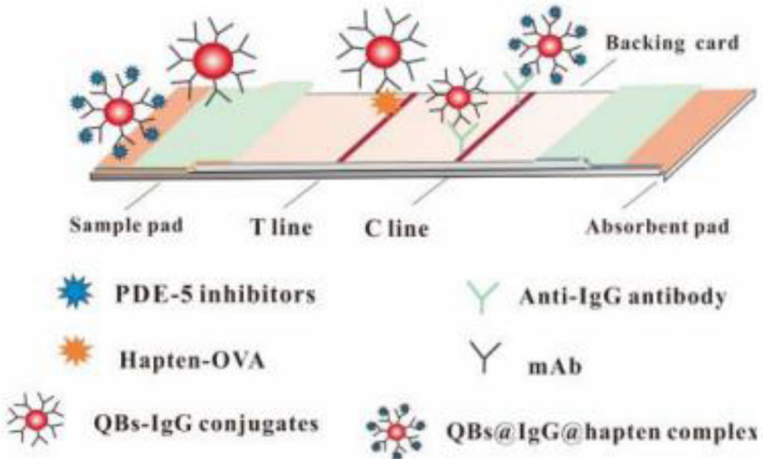


Fig. 2.
Schematic diagram of the QBs-LFIA

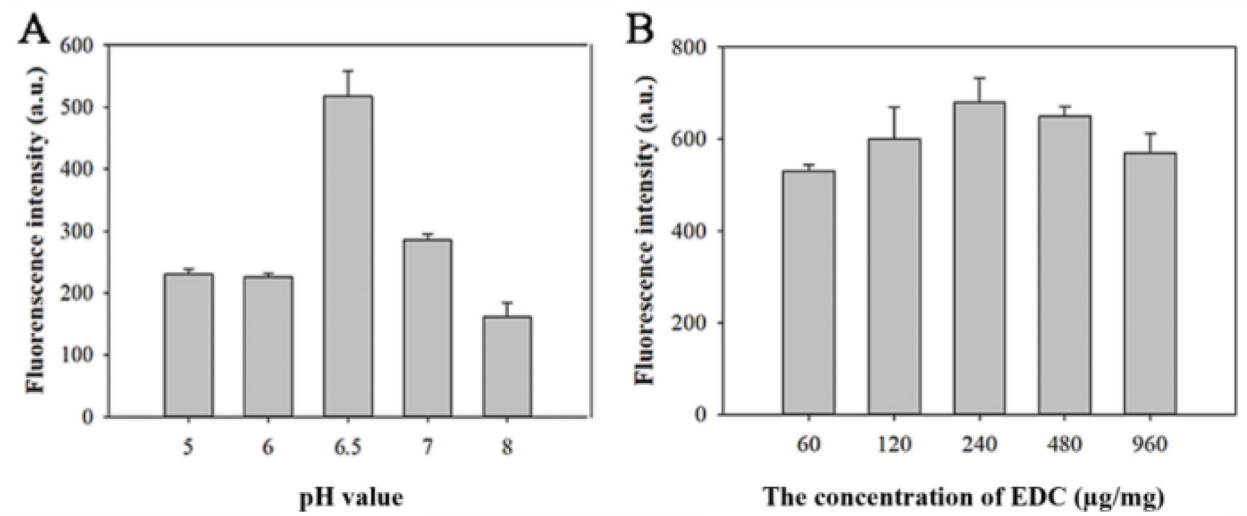


Fig. 3.

Optimization of the preparation of QBs-mAb probe. (A) The pH value. (B) The concentration of EDC.

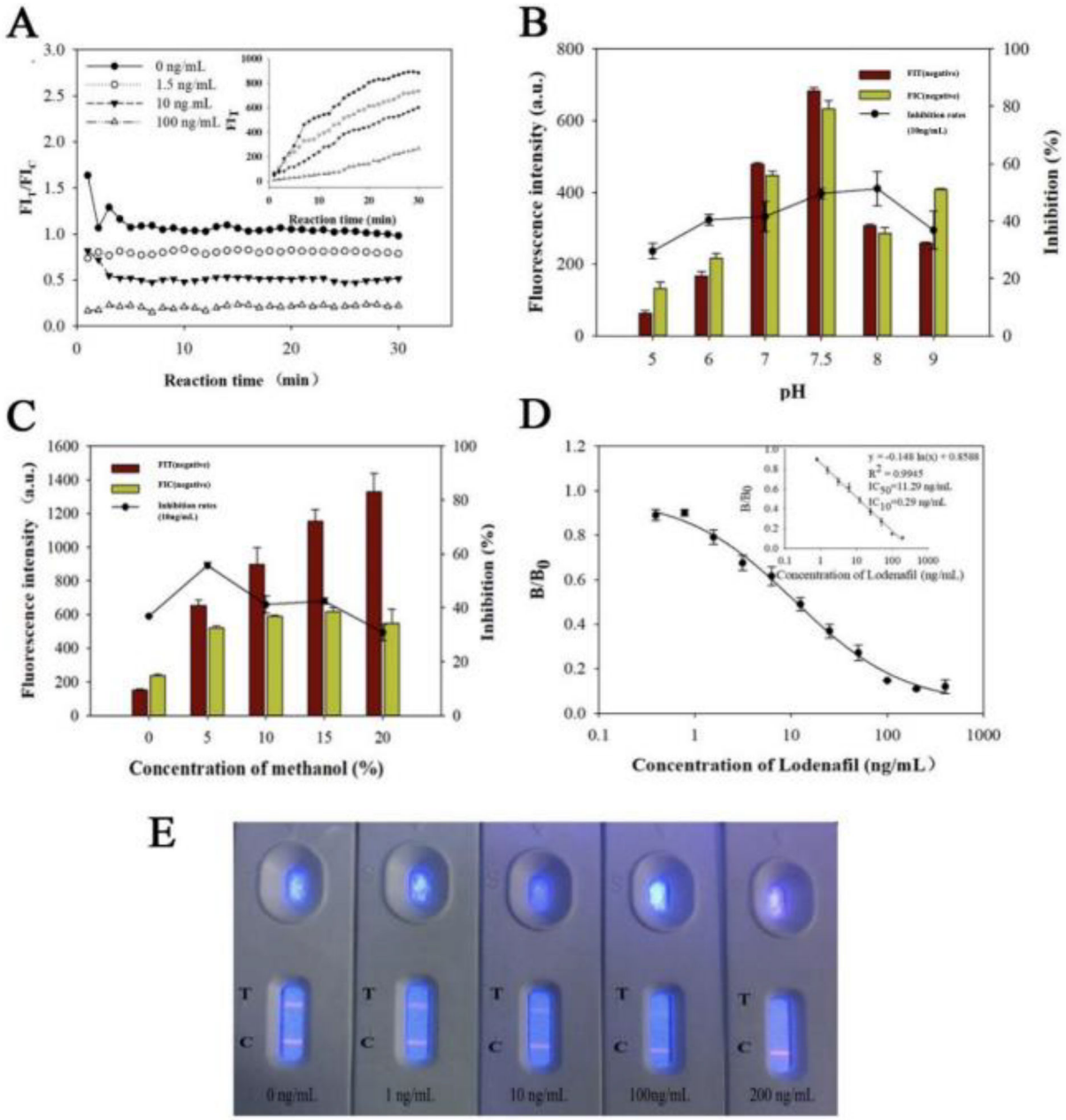


Fig. 4.

The optimization of QBs-LFIA and detection results. (A) Immunoreaction dynamics of FI_T and FI_T/FI_C ratios. (B) Effect of pH value on FI_T(negative), FI_C(negative) and inhibition rates(10 ng/mL). (C) The concentration effect of methanol. (D) The standard curve of lodenafil using QBs-LFIA. (E) QBs-LFIA detecting lodenafil under UV light.

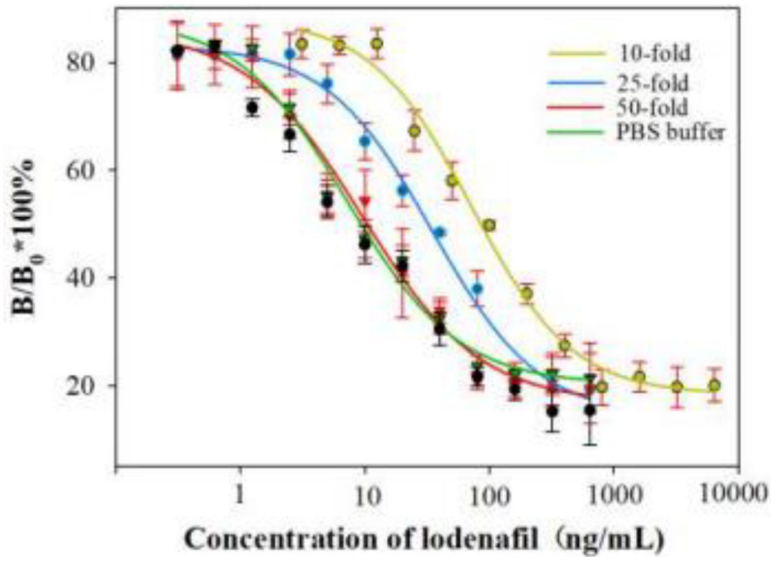


Fig. 5.
Dilution efforts on the elimination of matrix interference of spiked honey sample.

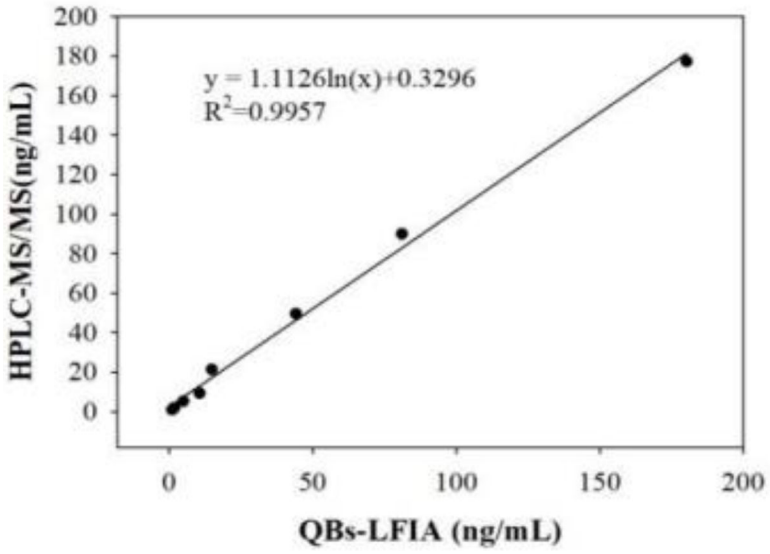


Fig. 6.

Correlation between QBs-LFIA and HPLC-MS/MS analysis for PDE-5 detection in honey samples.

Table 1.

Optimization of the concentrations of QBs-mAbs probe and LD-OVA using checkerboard strategy

NO.	The concentration of LD-OVA (mg/mL)	The volume of QBs-mAb (pL)	The Fl of test lines[a]	The Fl of control lines [a]	FIt/FIc	The inhibition rate (%)[b]
1	3	1	520114	341±27	1.5310.16	26.913.60
2	3	2	1038181	786±20	1.3210.14	9.6112.41
3	3	3	1534137	12121120	1.2710.10	29.5511.21
4	2	1	571151	473131	1.2110.20	3.4610.71
5	2	2	857121	67118	1.2810.01	13.4812.99
6	2	3	1579175	1271160	1.24±0.11	36.7513.72
7	1	1	506161	487175	1.04±0.03	44.8510.33
8[c]	1	2	670±22	550148	1.2310.25	54.1611.14
9	1	3	1271±263	14111289	0.9010.01	21.8712.85

[a]The means of the FI_T and Fl_C values are based on three duplicate measurements with the negative honey extract solution.

[b]The inhibition rates are obtained from the 10 ng/mL lodenafil-spiked sample.

[c]The optimal parameters of QBs-LFIA.

Table 2.

Sensitivity and CR of the QBs-LFIA for the determination of PDE-5 inhibitors.

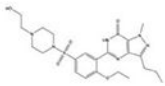
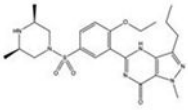
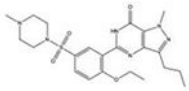
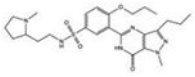
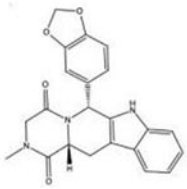
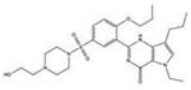
Compounds	Structure	IC_{50} (ng/mL)	IC_{10} (ng/mL)	CR (%)	Linear range(ng/mL)
Lodenafil		9.76	0.68	100	1.33–71.67
Methisosildenafil		21.17	1.09	46.10	2.29–195.35
Sildenafil		7.45	0.32	131.01	0.70–79.17
Udenafil		120.80	6.52	8.08	13.52–1079.03
Tadalafil		89.40	4.37	10.92	9.30–857.89
Mirodenafil		133.80	2.10	7.29	5.93–3014.12

Table 3.

Recoveries of inter-assay and intra-assay for the detection of six PDE-5 inhibitors in honey samples.

PDE-5 Inhibitors	Spiked concentration (ng/mL)	Intra-assay				Inter-assay[b]			
		Mean[a]	SD	CV (%)	Recovery (%)	Mean	SD	CV (%)	Recovery (%)
Lodenafil	1	1.02	0.02	2.81	102.40	1.08	0.13	12.71	108.16
	10	10.56	1.14	10.81	105.60	8.48	1.69	6.85	84.89
	100	81.01	2.01	2.49	81.01	90.44	5.93	6.56	90.44
Mirodenafil	3	2.26	0.19	8.77	75.33	2.19	0.19	8.77	75.33
	125	122.57	7.30	5.96	98.05	140.77	12.29	8.73	112.62
	3000	3450.68	211.33	6.12	115.02	2250.08	31.89	1.42	75.00
Methisosilden afil	2	1.97	6.16	3.12	98.61	1.83	3.11	16.90	91.61
	20	15.64	8.87	5.55	78.19	20.50	2.05	17.60	116.07
	200	173.73	8.54	4.92	86.87	154.32	6.54	4.24	77.16
Sildenafil	1	1.01	0.079	9.10	86.11	0.69	0.14	1.38	69.16
	5	4.71	0.13	2.83	94.16	6.09	0.39	7.95	96.71
	100	82.74	12.33	14.91	82.74	952.96	18.57	10.33	91.84
Udenafil	5	3.51	2.32	3.31	70.11	6.10	2.90	2.43	122.03
	50	64.37	5.90	9.17	128.75	57.68	11.56	10.02	115.37
	1000	1134.00	6.79	5.99	113.40	952.96	16.59	17.40	95.30
Tadalafil	5	4.15	0.45	10.76	83.19	4.40	0.72	1.63	88.06
	50	49.16	2.39	4.86	98.33	40.39	6.23	15.44	80.79
	800	910.86	99.04	10.87	113.86	1020.10	71.86	7.04	127.51

[a]Data are the mean ± SD at each spiked concentration of PDE-5 inhibitors (n=5).

[b]Assay was completed every 1 day for 3 days continuously (n=5).

Table 4.

Analysis of real samples (tablets, capsule and honey) by QBs-LFIA and HPLC-MS/MS (n=3).

Sample	Tablet (ng/mg)		Capsule(ng/mg)		Honey(ng/mL)	
No.	HPLC-MS/MS	QBs-LFIA	HPLC-MS/MS	QBs-LFIA	HPLC-MS/MS	QBs-LFIA
1	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
2	ND,ND,ND	- - -	10.15,9.85,8.86	± ± ±	ND,ND,ND	- - -
3	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
4	ND,ND,ND	- - -	7.39,7.05,6.98	± ± ±	ND,ND,ND	- - -
5	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
6	25.67,14.21,14.52	+ ± ±	ND,ND,ND	- - -	ND,ND,ND	- - -
7	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
8	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
9	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -
10	ND,ND,ND	- - -	ND,ND,ND	- - -	ND,ND,ND	- - -

[a]ND: not detectable. −:negative; +, ±: positive.