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Surface plasmon resonance biosensor for the detection of phenylethanolamine A in swine urine

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In the present study, an antibody against phenylethanolamine A (PEA) was produced, confirmed, and used in a surface plasmon resonance (SPR)-based measurement. Bovine serum albumin (BSA)-conjugated PEA was linked to nano-gold particles bound to L-cysteine modified on the surface of a Au-NP sensor chip. The concentrations of antigen and antibody were optimized, and the designed biosensor chip was investigated to examine the stability and accuracy of the proposed method. The recovery of PEA ranged from 80.4–93.4% in swine urine samples with spike levels of 5, 10 and 20 ng mL^{-1}, and the relative standard deviations of PEA were less than 2%. PEA analogues, such as clenbuterol, ractopamine, and salbutamol, did not influence the PEA measurement. The developed method could be used to measure PEA in swine urine samples.

Introduction

β-Agonists, a group of synthetic phenethylamines originally used to treat asthma, bronchitis, emphysema, and premature birth,[1,2] are also used as growth promoters in livestock rearing because of their redistribution effects,[3] such as increasing the lean meat content, increasing the fat breakdown, improving the feed conversion efficiency, and shortening the marketing time.[4,5] However, ingestion of β-agonists could cause several symptoms in humans, such as nausea, dizziness, increased heart rate, and muscle tremors.

Phenylethanolamine A (PEA) is a member of a family of β-agonists compounds, including clenbuterol (CL), ractopamine (LAC), and salbutamol (SAL), which have been illegally added to feed to promote growth.[6] Bulletin no. 1519 of the Ministry of Agriculture of China prohibits the use of PEA in feed and animal drinking water due to the potential risk to human health.

Many analytical methods, such as high-performance liquid chromatography (HPLC),[7,8] liquid chromatography-mass spectrometry (LC-MS),[9–14] gas chromatography-mass spectrometry (GC-MS)[15] and enzyme-linked immunosorbent assay (ELISA)[16,17] have been used to measure β-agonists in biological samples. However, few analytical methods have been developed to detect PEA. In 2010, a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method was used to detect PEA in biological samples provided by the Ministry of Agriculture of China. LC-MS/MS method was developed to measure PEA in tissue of swine[6] and swine muscle[18] and a reversed phase liquid chromatography tandem mass spectrometry with QuEChERS was built to determine phenylethanolamine A in animal hair, tissues and feeds.[19] A direct competitive ELISA was developed to detect PEA in urine samples.[20] The LC-MS/MS method is sensitive and reliable, but the expensive equipment, time-consuming sample pretreatment and the need for skilled operator limit its application. Recently, optical nanosensors have been used in the detection of food safety owing to their short response times, cheap, high sensitivity and simplicity.[21] Compared to large analytical instruments, SPR is relatively cheap and does not require specialized operators.

Surface plasmon resonance (SPR) is an advanced technique that can detect the interaction between a ligand and a target biomolecule on chips in a real-time manner,[22] which plays a key role in medicine and science. SPR is an electromagnetic wave spread along the surface of nanoparticles or thin metal layers. In the Kretchmann–Raether, total internal reflection setup is used to couple the excitation beam to the interface, typically includes a prism, the optical excitation of the surface plasmon can be reached, where p-polarized, collimated light beam undergoes total internal reflection at a glass-metal film–dielectric interface.[23] For the improvement of SPR sensing performance, gold nanoparticles (AuNPs) are commonly used nanomaterials due to their original properties such as strong adsorption ability, large specific surface area, good conductivity and biocompatibility.[22] SPR biosensor has been used to measure aflatoxin B_1,[21] brain natriuretic peptide,[22] microRNA (miRNA) and cancer cells on the basis of multiple signal amplification strategies,[24] DNA hybridization events,[25] human plasma samples,[26] histamine,[27] antibiotic ciprofloxacin.[28]

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The aim of the present study was to develop a SPR method to determine PEA in swine urine samples.

Materials and methods

Chemicals and reagents

Phenylethanolamine A (PEA), clenbuterol (CL), ractopamine (LAC), salbutamol (SAL), bovine serum albumin (BSA), ovalbumin (OVA) and 3,3,5,5-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO, USA). Tween-20 and sucrose were purchased from Sinopharm Chemical Reagent (Shanghai, China). Rabbit anti-mouse immunoglobulin G (IgG) was purchased from Beijing ZhongShan Gold Bridge Biotechnology Limited (Beijing, China). All other reagents used in the present study were of analytical grade and obtained from standard sources. Well-fed BALB/c female mice were purchased from the Laboratory Animal Center of Hebei Province (Shijiazhuang, China). A myeloma cell line of SP2/0 origin was produced by the Biology Institute of Hebei Academy of Sciences.

To generate the PEA stock solution, 10.0 mg of PEA was dissolved in 10 mL of methanol and stored at 4 °C. The stock was diluted with phosphate-buffered saline (PBS; sodium phosphate 10 mmol L^{-1}, pH 7.4) prior to use.

Instruments

The equipment included a surface plasmon resonance (SPR), AuNP sensor chip (Company of Zhonglongyicheng, Beijing, China) and a 3 K 15 centrifuge (Sigma, German). The water used in the experiment was obtained from a MilliQ purification system (Millipore, USA).

Conjugation of PEA to BSA and OVA

The BSA-PEA conjugates were generated as described by Bai *et al.*[20] A total of 2 g of PEA was dissolved in acetone, and the solution was refluxed for 5 hours after the addition of 0.8 g of bromopentaldehyde. The acetone was evaporated after the reaction, and the product was extracted by water and ethyl acetate. The crude product was obtained after evaporation and subsequently purified by column chromatography with methanol : dichloromethane (v/v, 1 : 5). The product was a half antigen. A total of 7 mg of the half antigen was dissolved in dimethylformamide, and the mixture was termed solution I; 30 mg of carbodiimide and 20 mg of *N*-hydroxysuccinimide were dissolved in 0.5 mL of deionized water, and then the mixture was added to solution I. Solution II was obtained after reaction at room temperature for 24 hours. A total of 50 mg of BSA was dissolved in 2 mL of phosphate buffer (0.01 M, pH 7.4) and slowly added dropwise to solution II. The reagent was reacted at room temperature for 24 hours. Subsequently, 34 mg of OVA was used in place of BSA, and the preparation was conducted in the same manner. When the reaction was completed, the mixture was dialysed with 0.01 mol L^{-1} PBS (pH 7.4). UV-Vis spectroscopy was used to determine the extent of conjugation.

Preparation and identification of the monoclonal antibody (MAb)

PEA-BSA was subcutaneously injected into the backs of five female BALB/c mice (6 to 8 weeks) at multiple sites. For the initial immunization, 0.15 mg of PEA-BSA was dissolved in 0.5 mL of NaCl solution (0.9%) emulsified with 0.5 mL of Freund's complete adjuvant. For subsequent injections, Freund's incomplete adjuvant was used in place of Freund's complete adjuvant, and immunization was performed with the same dose. This strengthening immunization was performed at 3 days prior to cell fusion with 0.3 mg of PEA-BSA dissolved in 0.5 mL of NaCl solution (0.9%).

SP2/0 myeloma were fused with spleen cells as described by Yang *et al.*[29] High-specificity and stable monoclonal cell strains against PEA were repeatedly screened out by confirmation detections. The cell strains were cultured to achieve a certain quantity and then injected into atoleine-pretreated BALB/c mice to produce ascites fluids. The ascites fluids were purified according to the octanoic acid-ammonium sulfate method. The PEA antibody was freeze-dried and stored at −20 °C, and the antibody was thawed and diluted with PBS prior to use.

Preparation of the Au-NP sensor chip

There are nano-gold particles connected to the L-cysteine that has been modified on the surface of the Au-NP sensor chip. Au-NP and L-Cys were assembled according to Yongle Ye's method.[30] Prior to modification, the glass slides were cleaned, immersed in 0.5% L-Cys acetic acid solution at 37 °C for 5 h and then rinsed multiple times with doubly-distilled water to eliminate physically adsorbed L-Cys, then soaked in 15 nm nanometer gold solution at 4 °C overnight, washed with doubly-distilled water and dried. Repeat the above steps. For the Au-NP sensor chip, the surface of the chip was washed with deionized water and dried by nitrogen. A total of 80 μL of 2.5 mg mL^{-1} PEA-BSA solution was dropped onto the Au-NP sensor chip, which was then incubated for one hour at 37 °C. The Au-NP sensor chip was washed with deionized water and dried under a nitrogen stream. To enclose bare sites to avoid non-specific adsorption, 3% BSA was added to surface of the Au-NP sensor chip, and the sensor chip was washed with deionized water after incubation for one hour at 37 °C. The Au-NP sensor chip could be used after nitrogen drying. The structure of the Au-NP sensor chip is shown in Fig. 1. PEA-BSA was adsorbed to Au-NP due to electrostatic and van der Waals interactions.[31]

Selection of the optimal antigen and antibody concentrations

In the experiment, PEA-BSA was conjugated to the sensor chip, and the antibody was added to the sample solution, which was used for measurements. Then, 10 μL of PEA-BSA solution at

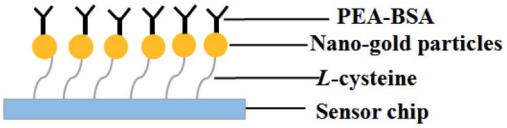

Fig. 1 The structure of the Au-NP sensor chip.

different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg mL^{-1}) was fixed onto the surface of the Au-NP sensor chip and incubated for one hour at 37 °C. Antibody solution at a concentration of 30 μg mL^{-1} was used to determine the optimal concentration of antigen.

Under the optimal antigen concentration, the antibody solution at different concentrations (10, 15, 20, 25, 30, 35 and 40 μg mL^{-1}) was evaluated to confirm the optimal concentration of antibody.

Regeneration and injection conditions

In order to investigate the regeneration of the sensor chip, 10 mmol L^{-1} Gly-HCl (pH 2.0–2.5), 50 mmol L^{-1} NaOH and 100 mmol L^{-1} NaOH were used as regeneration solutions, the instrument response was detected. The volume of regeneration solution was 100 μL and the flow rate of 400 μL min^{-1}. The elution results and regeneration number of the sensor chip were used to evaluate the regeneration effect. The injection volume was 200 μL, and the injection rate was 20 μL min^{-1}. A sketch map of the test is illustrated in Fig. 2. The reaction had three steps: the first step was binding (the analytes in sample solution bind with the antibody; the SPR response sharply declined), the second step was dissociation (after the binding step, analytes gradually dissociated from the antibody), and the third step was regeneration (when 100 mmol L^{-1} NaOH was used in the experiment, the antibody was thoroughly segregated with the analytes, and the SPR response returned to the original value).

Sample preparation and sample determination

A total of 5 mL of swine urine sample was centrifuged for 30 minutes at 9500 rpm, and 1 mL supernatant was diluted to 10 mL with PBS. 0.4 mL mixed sample was blended with antibody solution of concentration of 60 μg mL^{-1} of same volume, then the analyte was analysed.

200 μL sample was added to the sensor chip at a speed of 20 μL min^{-1}, the time of sample injection was 10 min, and then 100 μL 0.1 mol L^{-1} of NaOH was used as the regeneration solution at a speed of 400 μL min^{-1}. The time of sample analysis was 12.3 minutes, each sample was examined in triplicate, then average value was adopted.

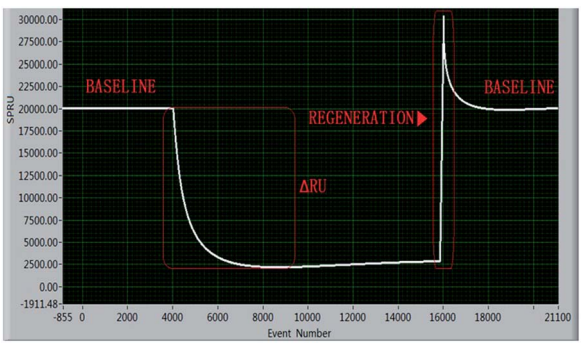

Fig. 2 The sketch map of the test.

Method validation

Standard curve establishment. Standard solutions of PEA with different concentrations (0.0, 2.5, 6.25, 12.5, 25.0 and 50.0 ng mL^{-1}) were prepared with an antibody concentration of 30 μg mL^{-1}. Standard solutions were tested to generate a standard curve.

Specificity determination. The chemical structures of RAC and PEA were similar, and the function of CL, RAC, and SAL was similar to that of PEA. As representatives, CL, RAC, and SAL were selected to evaluate the specificity. CL, RAC, and SAL were diluted to 5 ng mL^{-1}, and a blank control was used to determine the specificity of the sensor chip.

Accuracy and precision detection. Blank swine urine was supplied by the College of Veterinary, China Agricultural University, and samples with three spike levels (5, 10 and 20 ng mL^{-1}) were produced. Each sample was analysed in triplicate, and the recovery and variable coefficient were calculated. The spiked samples were evaluated by the standard LC-MS/MS method, and the values were used for comparison.

Results and discussion

Antigen synthesis identification

A UV-Vis spectroscopy method was employed to determine whether the link was successful. As shown in Fig. 3, the main peaks of BSA and PEA were both detected at 280 nm, and the superposition peak of the coupling was detected at 280 nm. In addition, a new absorption peak (–N=N–) at 360 nm was observed as the PEA-BSA peak. This peak indicated that the link was successful. According to the calculation, the coupling ratio of PEA-BSA was 15 : 1, and the coupling ratio of PEA-OVA was 17.4 : 1.

Antibody production and characterization

The characteristics of anti-PEA MAb were evaluated by indirect competition.[32,33] The competition between free PEA and PEA-

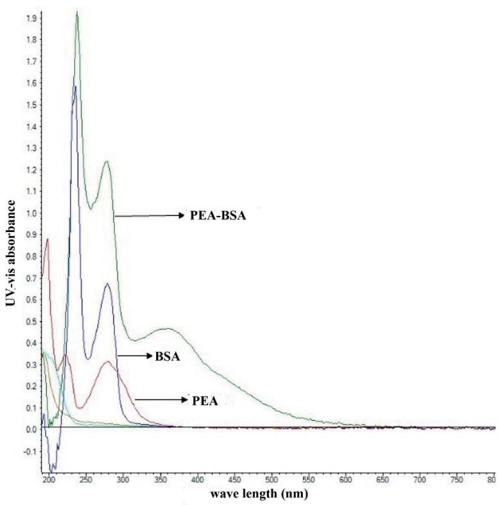

Fig. 3 UV-Vis absorbance of antigen.

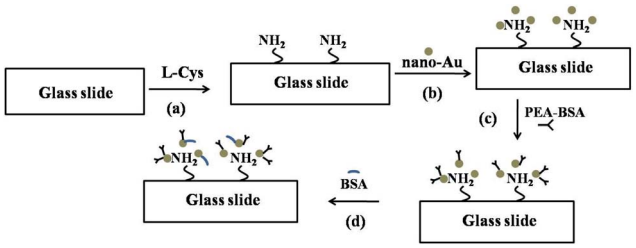

Fig. 4 The schematic illustration of the Au-NP chip modification process. (a) Self-assembled L-Cys; (b) absorption of nano-Au; (c) PEA-BSA loading; (d) blocking with BSA.

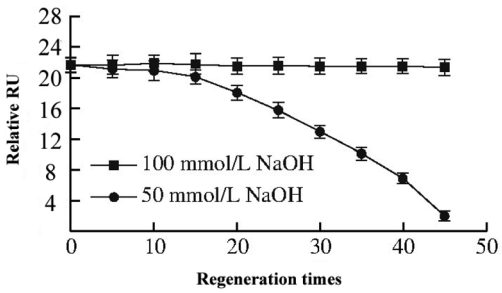

Fig. 5 Effect of regeneration cycles on Au-NP sensor chip response.

OVA for of MAb 1C11E4 was high, and the titre was determined to be 3.0×10^6. MAb 1C11E4 was used for subsequent experiments.

Characterization of the Au-NP sensor chip

The Au-NP sensor chip modification is brief described in Fig. 4. L-Cys could provide abundant active sites for binding analytes and tunable conductivity as an electrochemical sensor material. The glass slide was modified with Au-NPs to further enhance the sensitivity. According to Gao's method,[34] when L-Cys, nano-Au, PEA-BSA, BSA was modified on the surface of glass slide, the current on the surface of the glass slide decreased significantly, which was due to the fact that L-Cys, nano-Au, PEA-BSA, BSA covered on the surface of the glass slide, the film thickness increased, the difficulty of charge transfer gradually increased according. As the surface of the chip modified step by step, the film thickness increased, and the electrochemical impedance spectroscopy increased, which indicated the success of modification.

Reproducibility and stability of Au-NP sensor chip

Reproducibility and the sensing surface binding properties of the sensor were also the major concerns in evaluating the immunosensor, which completely depend on the regeneration capabilities and accuracy of detection results. As presented in Fig. 2, the SPR detection process is divided into the binding, dissociation and regeneration of the antibody in sample solution and antigen immobilized in the surface of sensor chip. The purpose of regeneration is to destroy the non-covalent binding between PEA-BSA and PEA antibody. When the solution of Gly-HCl under mild conditions (pH 2.0–2.5) was selected, PEA-BSA could not be completely separated from PEA antibody. Regeneration times of the chip and SPR response were investigate the regeneration effect when 50 mmol L^{-1} NaOH and 100 mmol L^{-1} NaOH were used as regeneration solution in the optimal PEA-BAS and antibody conditions.

As presented in Fig. 5, when 50 mmol L^{-1} NaOH was used as the regeneration solution, the activity of Au-NP sensor chip decreased rapidly when the regeneration times reached more than 20. However, when 100 mmol L^{-1} NaOH was used, the chip activity was still good and the instrument repeatability was good. So, 100 mmol L^{-1} NaOH was chosen as the regeneration solution. The sensor chip could be used 45 times with perfect activity in the condition of regeneration solution was 100 μL and the flow rate of 400 μL min^{-1}.

To ensure the quality of the chip, the chip should be stored at 4 °C when not in use and needs to be remodified or to change a now one if it is not used for more than one week.

The cost of excitation source, prism and the sensor chip is inexpensive, and the reproducibility of sensor chip is well, which reduce the cost of testing.

Selection of the optimal antigen and antibody concentrations

The immobilization concentration of the PEA-BSA conjugate (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg mL^{-1}) and the concentration of the antibody solution (10, 15, 20, 25, 30, 35 and 40 μg mL^{-1}) were optimized. In the absence of PEA, the sensor response reflects the specific binding between antigen and antibody. As presented in Fig. 6, the antibody solution was set as 30 μg mL^{-1}, as the increase of PEA-BSA concentration conjugated to the sensor chip, the response of instrument increased, but the concentration of the PEA-BSA was not the more the better, the excessive PEA-BSA on the surface of the chip could

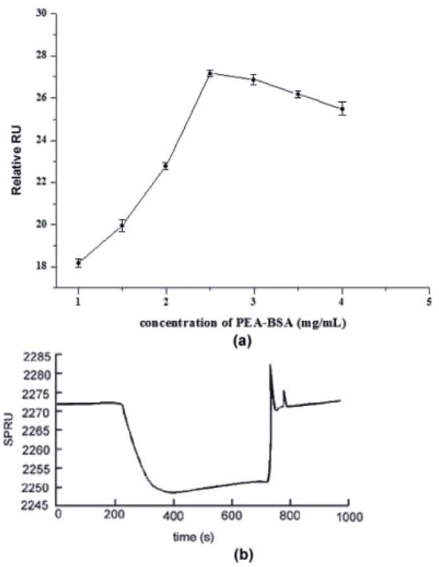

Fig. 6 Optimization of PEA-BSA. (a) The response of instrument with different concentration of PEA-BSA and antibody solution at a concentration of 30 μg mL^{-1}; (b) the SPR sensorgrams.

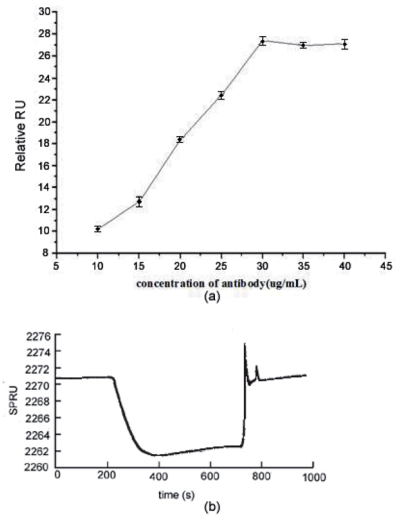

Fig. 7 Optimization of antibody. (a) The response of instrument with different concentration of antibody and PEA-BSA solution at a concentration of 2.5 mg mL^{-1}; (b) the SPR sensorgram.

generate steric hindrance which will prevent the recognition of specific binding site for antigen–antibody of PEA. In our experiment, the optimal PEA-BSA concentration was chosen as 2.5 mg mL^{-1}. As shown in Fig. 7, the binding value increased with the concentration of antibody increase, the selection of antibody depending on the antibody fixed on the chip surface and the amount of consumption. When the antibody concentration reaches the maximum binding of epitopes, the instrument response will reach the peak, then the sensor response basically remains at a similar level with the increase of antibody concentration. The amount of antibody should be appropriate, the detection limit of the instrument and method will rise when concentration of antibody is too large, and the linearity range is narrow when the concentration of antibody is too low which inhibit the use of the method. Considering the production cost of PEA antibody, high antibody consumption will increase the cost of detection. In conclusion, the optimal concentration of the antibody solution was chosen as 30 μg mL^{-1}.

Thus, the concentration of PEA-BSA conjugated to the sensor chip was confirmed to be 2.5 mg mL^{-1}, and the concentration of antibody added to the sample solution was fixed at 30 μg mL^{-1} in further experiments.

Method validation

Specificity of the method. As described, 5 ng mL^{-1} solutions of CL, RAC, and SAL were used to determine the specificity of the method, and no significant signal change was found. This finding indicates that the specificity of the method was perfect, and PEA analogues did not influence the measurement.

Method validation, including recovery and precision. Three concentrations (5, 10 and 20 ng mL^{-1}) of PEA spiked in the blank swine urine samples were used to determine the recovery. The PEA recovery values ranged from 80.4–93.4% in the swine urine samples. The recovery range conforms to the national standard of China GB/T 27404-2008 "Criterion on quality control of laboratories-chemical testing of food". The responses of the blank and spiked samples are presented in Fig. 8, and the vertical axis indicates the relative RU, which is the difference between the initial and the test values.

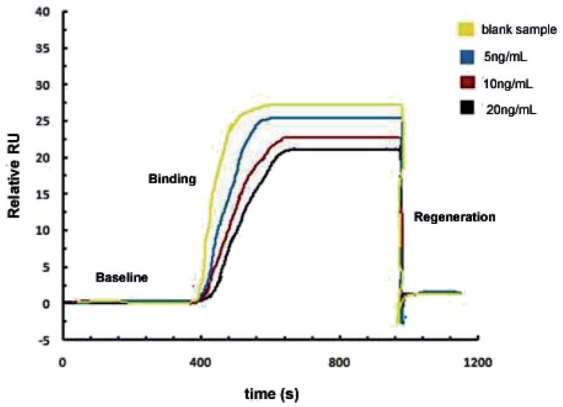

Fig. 8 The instrument responses of blank sample and spike samples.

The precision of the method was examined by performing five consecutive extractions ($n = 5$) of the spiked samples. The precision of the method was expressed as the relative standard deviation (RSD). The RSDs of PEA were less than 2%. The recovery and RSDs of the method are presented in Table 1. Comparing the SPR results with the LC-MS/MS results indicated good correlation between the two methods.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ). The SPR sensor used for sensitive quantification of PEA detection was characterized by a competitive reaction between the immobilized PEA-BSA conjugate on the Au-NP chip and the analyte. In the presence of PEA, the PEA-BSA would compete with the analyte binding to the specific PEA antibody. With the increase in the analyte concentrations, the relative RU correspondingly decreased, which were in complete agreement with the decrease in the free PEA antibody left. The response in an immunological system based on the antigen–antibody interaction is very commonly related to the logarithm of the antibody concentration. However, linear relationship also exist within certain range. The sensitivity and linear range of the method are affected by the concentration of conjugated antigen and antibody in the test solution. High concentration of antigen and antibody lead to the wide linear range and low method sensitivity, the difference of instrument response is not obvious when the concentration of analyte is low. While low concentration of antigen and antibody lead to the narrow linear range and high method sensitivity, the instrument response could be clearly distinguished with low concentration of analyte.

The method was linear in the concentration range between 0 and 50 ng mL^{-1}, the relative RU of the instrument was defined as y, and the concentration of PEA solution was defined as x, with a linear equation of $y = -0.259x + 27.323$. The correlation coefficient (r^2) of the linear equation was greater than 0.98. The LOD of the method was 1.39 ng mL^{-1}, and LOD was defined as the analyte concentration providing three times the standard deviation of the signal-to-noise ratio of the blank sample. The LOQ of the method was 5 ng mL^{-1}, and LOQ was defined as the

Table 1 Recovery and precision

	SPR				LC-MS/MS			
Spike level (ng mL^{-1})	Measured value (ng mL^{-1})	Recovery (%)	Average recovery (%)	RSD (%)	Measured value (ng mL^{-1})	Recovery (%)	Average recovery (%)	RSD (%)
5	4.13	82.6	82.5	1.78	4.21	84.2	84.4	1.85
	4.02	80.4			4.35	87.0		
	4.17	83.4			4.15	83.0		
	4.09	81.8			4.23	84.6		
	4.21	84.2			4.17	83.4		
10	8.91	89.1	89.3	1.39	9.05	90.5	90.5	1.52
	8.86	88.6			8.96	89.6		
	8.79	87.9			9.15	91.5		
	8.95	89.5			8.87	88.7		
	9.12	91.2			9.21	92.1		
40	36.04	90.1	92.0	1.44	37.11	92.8	93.8	1.55
	36.50	91.2			38.15	95.4		
	37.36	93.4			37.53	93.8		
	37.14	92.8			38.02	95.0		
	36.96	92.4			36.79	92.0		

analyte concentration providing ten times the standard deviation of the signal-to-noise ratio of the blank sample.

Sample analysis

The developed method was used to evaluate PEA in twenty swine urine samples supplied by the College of Veterinary, China Agricultural University, and PEA was detected in one sample at a concentration of 10.2 ng mL^{-1}. The positive sample was confirmed by LC-MS/MS to have a concentration of 10.7 ng mL^{-1}, which indicated that the method could be used for sample analysis. Abnormal sensor grams will be observed when a bubble enters the system, and a wash is necessary when abnormal sensor grams are obtained.

Conclusions

In this experiment, a PEA antibody was produced and confirmed, and the antibody was used in the SPR determination. As a vector, nano-gold was used to bind PEA to the surface of the SPR biosensor chip. The concentrations of antigen and antibody were optimized, and the designed biosensor chip was used to examine the stability and accuracy of the proposed method. The recoveries of PEA ranged from 80.4–93.4% in swine urine samples for spike levels of 5, 10 and 20 ng mL^{-1}, and the RSDs of PEA were less than 2%. Analogues of PEA, such as CL, RAC, and SAL, did not influence the PEA measurement. Compared with articles published,[6,18–20] the advantages of this method lies in its simple pretreatment process and easy operation. The developed method was successfully used to measure PEA in swine urine samples and could be used to determine whether β-agonists exist in pork meat.

Ethical statement

Use and handling of animals were strictly in accordance with the review guidelines of Animal Ethical and Welfare of Hebei Food Inspection and Research Institute. The experiment was carried out according to the guidelines of the National Institutes of Health for Care and Use of Laboratory Animals and was approved by Animal Ethical and Welfare Review Committee of Hebei Food Inspection and Research Institute.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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