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Preparation of Polyclonal Antibodies and Development of a Direct Competitive Enzyme-Linked Immunosorbent Assay To Detect Residues of Phenylethanolamine A in Urine Samples

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ABSTRACT: Phenylethanolamine A (PEAA) is a phenethanolamine member of the family of β-adrenergic agonists (β-agonists) compounds. To determine PEAA residues, we established a rapid direct competitive enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody produced with the immunogen PEAA−HSA conjugate. The antibody showed high sensitivity, where IC_{50} and the limit of detection were 0.3 and 0.02 μg/L, respectively. The specificity of the assay was evaluated by the measurement of cross-reactivity of the antibody with 15 β-agonists compounds. The data demonstrated that the antibody was highly specific for PEAA, with negligible cross-reactivity (CR) with other β-agonists compounds (CR < 0.1%) including ractopamine (CR is 0.3%). Recovery rates ranged from 81% to 110%, indicating relatively good parallelism and accuracy of the assay when applied to real samples. The detection limit in blank urine samples was 0.5 μg/L. The coefficient of variation was below 18% and 20% for intra-assay and inter-assay, respectively, demonstrating an acceptable level of precision. Largely consistent results were obtained for the urine samples by ELISA and UPLC−MS/MS methods. From a practical point of view, the prototype kit could be advantageously used for the screening of large groups of urine samples, and the kit employed has reliability even in routine application for the control of the illegal use of the drug.

KEYWORDS: *phenylethanolamine A, β-agonists, ELISA, polyclonal antibody, residues*

INTRODUCTION

β-Agonist compounds are widely used as growth promoters in livestock production. Feeding animals with β-agonists can bring many advantages, such as the promotion of repartitioning of fat into muscles, increasing average daily weight gain, improving the efficiency of feed conversion, saving feed, and shortening the time to market if higher doses are administered.[1−4] However, meat products obtained from illegally treated animals with these compounds may pose potential risks linked to adverse cardiovascular and central nervous system effects.[5] Because of these potential risks for human health, ractopamine hydrochloride (RAC·HCl, MW 337.85 g/mol, (1*R**,3*R**), (1*R**,3*S**)-4-hydroxy-*R*-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino] methyl]-benzenemethanol hydrochloride), as a typical member of the family of β-agonists, is used in a restricted manner within a low limit of dosage in many countries, but banned in some countries, while clenbuterol has been prohibited from being used in livestock production in most countries. Unfortunately, although the governments have made great efforts to strengthen the supervision and monitor the use of β-agonists, new alternatives emerge and bring more harm on livestock production and human health, such as phenylethanolamine A.

Phenylethanolamine A [PEAA, $C_{19}H_{24}N_2O_4$, MW 344.17 g/mol, 2-(4-(nitrophenyl) butan-2-ylamino)-1-(4-methoxyphenyl)ethanol] is a phenethanolamine member of the family of β-agonists. It is a synthetic substance and the isomer of formoterol (which also belongs to the family of β-agonists), and structurally similar to ractopamine (see Figure 1). Since 2010, PEAA has been prohibited from being used in

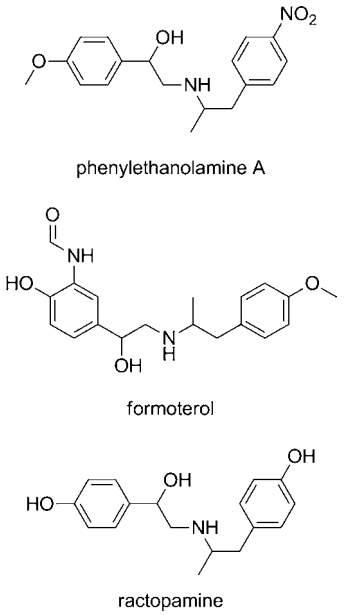

Figure 1. Chemical structures of PEAA and some closely related compounds.

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feeds and animal drinking water in bulletin no. 1519 issued by the Ministry of Agriculture of China. Although many analytical methods have been reported to monitor other β-agonists (like ractopamine and clenbuterol) in animal feeds, urine, and tissues using instrument methods like high-performance liquid chromatography (HPLC) with chemiluminescence or electrochemical detection, LC-fluorescence, liquid chromatography–mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS),[6−11] and immunochemical approaches,[12,13] seldom analytical methods have been developed to monitor PEAA currently. In 2010, the Ministry of Agriculture of China has issued a standard for the detection of PEAA in feed using high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) detection method. In addition, Sun et al. reported a method to detect PEAA residues in porcine muscle tissue according to the LC–MS/MS method.[14] As far as we know, no other related reports have been published. However, the current analytical approaches using instrument methods for PEAA are complicated, time-consuming, and expensive; on the other hand, integral production chain systems currently demand faster onsite (farmhouses) and/or online (slaughterhouses) test systems. Therefore, it is necessary to develop a more rapid, sensitive, effective, and convenient method to meet the needs of the current situation, and the ELISA technique becomes the most potential candidate.

Recently, a PEAA ELISA test kit format has become commercially available (Shenzhen Lvshiyuan Biotechnology Co., Ltd., Shenzhen, China), which is based on an indirect competitive enzyme immunoassay. Differently, the objective of our study is to develop a direct assay for the determination of PEAA residues in pig urine samples. The direct format needs fewer assay steps than the indirect competitive ELISA, and then is more rapid and convenient for screening of large groups of samples. In this Article, the polyclonal rabbit antibodies, which were produced with the synthesized immunogen PEAA-HSA, were highly sensitive to PEAA. Our work shows the advantages of good specificity, high sensitivity, and adequate reliability in urine sample of PEAA residues.

MATERIALS AND METHODS

Reagents. Horseradish peroxidase (HRP), ovalbumin (OVA), human serum albumin (HSA), goat antirabbit IgG HRP conjugate, Tween 20, glycerol, and Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Co., Beijing, China. Skim milk was purchased from Becton-Dickingson Co., U.S. Terbutalin sulfate, salbutamol, ractopamine hydrochloride, clenbuterol hydrochloride, and zilpaterol hydrochloride were from Dr. Ehrenstorfer Co., Germany. PEAA was supplied by Hangzhou DNA Sci-Tech Co. Ltd., China. PEAA-D_3, cimaterol, tulobuterol hydrochloride, penbutolol hydrochloride, clorprenaline hydrochloride, brombuterol hydrochloride, cimbuterol, and mabuterol hydrochloride were from Witega Co., Germany. Fenoterol hydrochloride, bambuterol, and formoterol were from EP Co. 3,3′,5,5′-Tetramethylbenzidine (TMB), sodium periodate, ethylene glycol, zinc powder, and H_2SO_4 were purchased from Beijing Chemical Reagent Factory, China. Deionized water was purified on a Milli-Q system (Millipore, MA). All other solvents and chemicals were analytical reagents and used without further purification.

Buffers and Solutions. The following buffers and solutions were used in the experiments: (1) 50 mmol/L carbonate/bicarbonate buffer (pH 9.6) was used as a coating buffer; (2) 10 mmol/L PBS containing 0.05% Tween 20 was used as a washing buffer; (3) washing buffer containing 5% skim milk was used as a blocking buffer; (4) 10 mmol/L PBS (pH 7.0) was used as the buffer of standard solutions; (5) 1 mol/L H_2SO_4 was used as a stopping reagent.

Instrumentation. ELISA was performed in polystyrene 96-well stripwell microplates (Costar, Cambridge, MA) and spectrophotometrically read with an automatic microplate reader Tecan Sunrise, Switzerland. UV–vis data were collected on a U-3010 spectrophotometer from Hitachi Co. Centrifugation was carried out with a centrifuge from Thermo Electron Corp. Protein dialyses were performed using membra-cel dialysis tubes from Viskasecompanies Inc., U.S.

Preparation of Immunogen and Coating Antigens. As Figure 2 shows, the immunogen of PEAA-HSA was prepared according to the

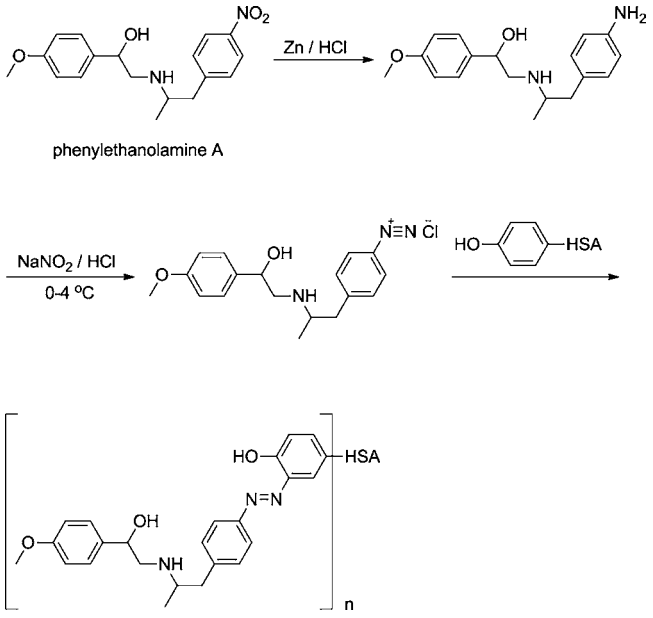

Figure 2. Synthetic procedure for immunogen of PEAA–HSA through the diazotization reaction.

diazotization reaction. For the reduction of $-NO_2$ group in PEAA molecule, 30 mg of PEAA was dissolved in 3 mL of 1 mol/L HCl, and then 10 mg of zinc powder was added. The mixture was stirred slowly for several hours at room temperature. The obtained solution was cooled to 0–4 °C using an ice–water bath for preparation of the PEAA–HSA conjugates. 0.5 mL of 0.15 mol/L $NaNO_2$ was added into solution dropwise, and then the pH value of the mixture was adjusted to 1.5 with 1 M HCl. After the mixture was stirred for 2 h at 0–4 °C, the excessive $NaNO_2$ was removed using ammonium sulfamate, and the pH value of the mixture was readjusted to 7.0 with 2 mol/L NaOH. Finally, the mixture was added dropwise into 4 mL of PBS solution containing 200 mg HSA, and the mixture was allowed to react overnight at 4 °C to prepare the immunogen PEAA–HSA. The reaction mixture was dialyzed under stirring against PBS (10 mmol/L, pH 7.4) for 3 days with frequent changes of PBS solution to remove the uncoupled free hapten. The obtained PEAA–-HSA conjugate was stored at −20 °C for future use. A UV–vis absorbance method was employed to determine whether the linking had been a success, and further to estimate the hapten/protein ratio (Figure 3).

The coating antigen of PEAA–OVA conjugate was prepared by a similar method.

Preparation of PEAA–HRP Conjugate. PEAA–HRP conjugate was prepared using the $NaIO_4$ method. First, $-NO_2$ of PEAA molecule was reduced using the above method. In detail, 10 mg of PEAA was dissolved in 1 mL of 1 mol/L HCl, and then 4 mg of zinc powder was added. The mixture was stirred at room temperature for about 4 h. After the solution was cooled to 0–4 °C, the excessive zinc powder was removed by a filter, and the pH value of the mixture was adjusted to 5–6 with 2 mol/L NaOH. This resulting solution was named Solution A. Second, 10 mg of HRP was dissolved in 1 mL of

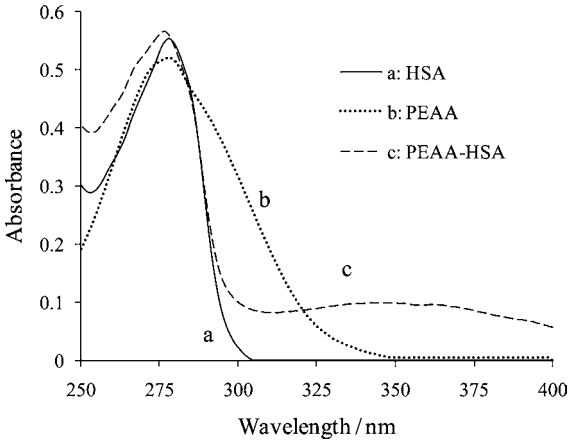

Figure 3. UV−vis spectrum: (a) HSA, (b) PEAA, and (c) PEAA−HSA.

purified water, and 100 μL of a freshly prepared sodium periodate solution (60 mmol/L) was then added. This mixture was incubated for 30 min at 4 °C in the dark. A total of 1 μL ethylene glycol was added to stop the reaction. After an additional incubation for 30 min at room temperature, the above Solution A was added into the HRP mixture while stirring, and the conjugation mixture was incubated at 4 °C overnight. The formed PEAA−HRP conjugate was dialyzed under stirring against PBS (10 mmol/L, pH 7.4) for 3 days with frequent changes of the PBS solution. Next, the PEAA−HRP conjugate was diluted with an equal volume of glycerol and stored at −20 °C.

Immunization of Rabbits. Animal welfare and experimental procedures were carried out strictly in accordance with the "Guidelines for Ethics Review on Laboratory Animal Welfare of Beijing" (2005) and the "International Guiding Principles for Biomedical Research Involving Animals" (1985), and was approved by Animal Care and Use Committee of China Institute of Veterinary Drug Control. All efforts were made to minimize the animals' suffering and to reduce the number of animals used. Three Japanese white rabbits, which had been purchased from Beijing laboratory animal research center, were immunized by subcutaneous injection with immunogen PEAA−HSA. The initial immunization was subcutaneously injected at multiple sites on the back of rabbits with 1.0 mg of immunogen conjugate in 0.5 mL of NaCl solution (0.9%) and 0.5 mL of Freund's complete adjuvant. The second immunization was carried out 4 weeks later by intramuscular injection on two hind legs with 0.5 mg of immunogen conjugate in 0.25 mL of NaCl solution (0.9%) and 0.25 mL of Freund's incomplete adjuvant. Subsequent immunizations were performed at 2 week intervals in the same way. The booster immunizations were performed for 5−10 times in all. Seven days after the last boost without any adjuvants, all rabbits were exsanguinated, and the sera were collected. The crude serum obtained was purified by the saturated ammonium sulfate (SAS) precipitation method [purified three times using 50%, 33%, and 33% (v/v) of SAS, respectively], and then dialyzed against PBS (10 mmol/L, pH 7.4). The purified serum was aliquotted before being stored at −40 °C.

Antisera Evaluation. Indirect competitive ELISA was developed for assessing the sensitivity of the serum from each animal to PEAA. Microplate wells were coated with 150 μL per well of PEAA−OVA conjugate (1 μg/mL) diluted in 0.05 M carbonate−bicarbonate buffer (pH 9.6) by incubation overnight at 4 °C. The plate was washed three times with washing buffer and blocked with 5% skim milk at room temperature for 1 h. After the blocking solution was removed, the plate was dried at room temperature and 30% relative humidity for 4 h. PEAA standard solutions (0, 0.1, 1, and 10 μg/L, volume: 50 μL) were added to each well followed by the addition of 50 μL of diluted antisera (1:5000), and the plate was incubated for 1 h. The plate was washed three times. The diluted goat antirabbit IgG HRP conjugate (100 μL) in washing buffer was added to each well. The plates were incubated at room temperature for 30 min and washed three times. The substrate solution TMB (0.1 mg/mL, 100 μL) was added to each well and incubated at room temperature for 15 min. The reaction was stopped by the addition of aqueous H_2SO_4 (1 mol/L). The optical density (OD) at a wavelength of 450 nm in each well was read using an ELISA reader. The result was expressed in $B/B_0 \times 100\%$ (%), where B is the mean absorbance of the wells containing competitor and B_0 is the mean absorbance of the wells without competitor. The percent inhibition is expressed as follows:

$$\text{inhibition (\%)} = [1 - (B/B_0)] \times 100\%$$

Direct Competitive ELISA. A competitive enzyme immunoassay method for PEAA has been developed using an antiserum raised in rabbits by immunization against the PEAA−HSA. The 96-well microplate was precoated with diluted anti-PEAA polyclonal antibody (1:10 000) overnight at 4 °C in a coating buffer. The wells were washed three times with washing buffer and blocked with 5% skim milk at room temperature for 1 h. After the blocking solution was removed, the plate was dried at room temperature and 30% relative humidity for 4 h. PEAA standard solutions (50 μL) with 100 μL of PEAA−HRP conjugate (0.1 mg/L) were added to each well. The plate was then incubated for 30 min at room temperature. After washing the wells three times, the substrate solution TMB (100 μL) was added to each well and incubated at room temperature for 15 min. The reaction was stopped by the addition of aqueous H_2SO_4 (1 mol/L). The optical density at a wavelength of 450 nm in each well was read using an ELISA reader.

Specificity Determination. The specificity of the antibody was evaluated by measuring percent cross-reactivities (CR, %), which were determined by measuring the IC_{50} (50% inhibitory concentration) values using PEAA and the other 15 β-agonists compounds (including clenbuterol, ractopamine, terbutalin, cimaterol, fenoterol, tulobuterol, salbutamol, penbutolol, clorprenaline, brombuterol, mabuterol, zilpaterol, cimbuterol, bambuterol, formoterol) as competitors in the competitive direct ELISA described above. The concentrations of PEAA standard solutions were 0, 0.05, 0.15, 0.45, 1.35, and 4.05 μg/L, and those of the other 15 β-agonists standard solutions were 0, 1.6, 8, 40, 200, and 1000 μg/L. The CR values were calculated from the following formula:

$$\text{CR (\%)} = (IC_{50,PEAA}/IC_{50,competitor}) \times 100\%$$

ELISA Validation. Immunoassay validation was carried out using the limit of detection (LOD), the recovery (%) of the fortified PEAA, and coefficients of variation (CVs). Some pig urine samples, which were collected from the slaughterhouses and certified as free of PEAA using the LC−MS/MS method, were used as blank samples in this study. The determination of LOD for real samples in this assay was based on 20 blank urine samples. The concentrations of PEAA were calculated according to a standard curve (0, 0.05, 0.15, 0.45, 1.35, and 4.05 μg/L), as well as the mean value for 20 blank urine samples. The LOD was calculated to be the sum of the mean value and 3 times that of the SD (standard deviation) value (mean value +3SD).

The blank urine sample was fortified with PEAA to give the final concentrations at 0.5, 1.0, and 1.5 μg/L, and then centrifuged at 5000 rpm at 25 °C for 5 min. The supernatants without dilution were used for ELISA. The direct competitive enzyme immunoassay method was the same as mentioned above, and the volume of added samples was 50 μL. Sample recoveries were determined from a standard curve and calculated using the equation: recovery rates (%) = measured concentration/fortified concentration × 100%. The precision of the ELISA was analyzed by repeated determination of the intra- and inter-assay CVs of the sample at the levels of 0.5, 1.0, and 1.5 μg/L. Inter-assay variation was calculated from the analysis of six replicates carried out on three different days. Intra-assay variation was measured by analysis of six replicates on a single day.

Comparison of ELISA and LC−MS/MS Methods. Four pigs were given a free choice access to feeds (with the concentration of phenylethanolamine A 100 mg/kg) for two days, and then their urine samples were collected. The concentration of phenylethanolamine A in these urine samples was analyzed using the ELISA method in this

assay and UPLC−MS/MS, respectively. Because of the high concentration of PEAA in urine samples, these samples were diluted 50 times before detection using ELISA method.

The sample preparation procedure for LC−MS/MS measurement was developed from the method for determination of ractopamine in swine tissues.[15] Briefly, appropriate amounts of internal standard (PEAA-D_3) solution were spiked into an aliquot of 2.0 g of urine sample before adding 6 mL of 0.2 mol/L ammonium acetate and 40 μL of β-glucuronidase/aryl sulfatase. After being incubated overnight for 16 h at 37 °C, the sample was adjusted to pH 9.5−10.0 using 10 mol/L NaOH in water and extracted with 20 mL of ethyl acetate. The supernatant was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 0.5 mL of 10% methanol in water. Finally, the solution was filtered (0.2 μm pore size) and injected on the UPLC−MS/MS system.

Chromatographic separations were performed on an ACQUITY BEH C_{18} column (50 × 2.1 mm i.d., 1.7 μm particle size; Waters, Milford, MA) using an ACQUITY UPLC system (Waters, Milford, MA) equipped with a binary solvent delivery system and an autosampler. The mobile phase was constituted by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). While containing a constant flow rate of 0.3 mL/min, the gradient elution program was: 0−1.0 min, 5% B; 1.0−5.0 min, a linear gradient from 5% to 60% B; 5.0−6.0 min, 95% B; 6.0−8.0 min, 5% B.

The UPLC system was coupled to a tandem mass spectrometer (Micromass Quattro Premier XE, Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source. The parameters used for the mass spectrometer were as follows: ionization mode, electrospray positive; capillary voltage, 3.0 kV; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas (N_2) flow rate, 50 L/h; desolvation gas (N_2) flow rate, 650 L/h; collision cell pressure, 3.8 × 10^3 mbar. MS/MS acquisition was performed using multiple reaction monitoring (MRM). Optimized acquisition parameters of PEAA and PEAA-D_3 are given in Table 1.

Table 1. Mass Spectrometry Parameters for Detection of PEAA and PEAA-D_3

analyte	precursor ion (m/z)	product ions (m/z)	cone voltage (V)	collision energy (eV)
PEAA	345	327	20	15
		150		25
PEAA-D_3	348	330	20	15

RESULTS AND DISCUSSION

Immunogen and Coating Antigen Conjugation. As a small molecule with a molecular mass of 344.17 g/mol, PEAA is not able to elicit the immune response of an animal to produce the anti-PEAA antibody and is therefore nonimmunogenic. To make it immunogenic, it must be conjugated to a carrier protein before immunization. PEAA was conjugated with HSA according to the diazotization reaction (see Figure 2). First, the $-NO_2$ group of PEAA molecule was reduced to $-NH_2$ by zinc powder in acidic condition. The generated $-NH_2$ group would be transformed into diazo group by HNO_2 ($NaNO_2$ and HCl). When the protein HSA added, the diazonium group of PEAA would attack toward the electron-rich points on the target molecules.[16] Take the main reaction between the diazonium group of PEAA with tyrosine residues of HSA as an example. It is known that phenolic compounds are modified at ortho and para positions to the aromatic hydroxyl group. However, when electron effects and steric effects are concerned, the diazo reaction usually proceeds at the para position of the aromatic hydroxyl group. When the para position of the aromatic hydroxyl group has a substituent, the reaction conducts at the ortho position.[17] For tyrosine side chains of HSA, only the ortho modification is possible (see Figure 2).

To obtain evidence of successful conjugation, the UV−vis spectra recorded from 250 to 400 nm were measured for HSA, PEAA, and PEAA−HSA conjugate, as shown in Figure 3. HSA has an absorbance peak at 278 nm, which comes from conjugated double bonds of tryptophan and tyrosine residues in the molecule (curve a).[18] PEAA has a broader absorbance peak at 278 nm resulting from the conjugated system of benzene rings and the chromophore $-NO_2$ group (curve b, in Figure 3). In the conjugate of PEAA−HSA, the nitrogen double bonds, working as bridges between the haptens and the protein carrier, connected several conjugation systems together to form a larger one and caused the shift to longer wavelengths, as shown in the synthetic procedure of PEAA−HSA in Figure 2. Consequently, a new absorbance band ranging from 330 to 380 nm was found for the conjugate of PEAA−HSA (curve c, in Figure 3). In addition, referring to the procedure by Jiang et al.,[19] an average hapten/protein conjugation ratio was confirmed as 15 for the conjugate of PEAA−HSA.

The corresponding coating antigen PEAA−OVA was prepared according to the same reaction, and gave a similar pattern in UV−vis spectrometry (data not shown).

Characterization of PEAA Polyclonal Antibodies. Table 2 shows the comparison of inhibitory effect of polyclonal antisera from different rabbits produced with PEAA in indirect ELISA. It is found that the individual differences of animals cause the differences of inhibitory effect. With the concentration of PEAA increases, the values of B/B_0 decrease for all of the antisera. It is also noted that at the same concentration of PEAA, the value of B/B_0 of antiserum R_3 is lower than that of other antisera, which means the percent inhibition is the highest in all of the antisera. Indirect ELISA was used to screen the antisera, and then our efforts were focused on direct ELISA based on the most sensitive antiserum R_3, which was selected for further characterization with regard to its high sensitivity.

Table 2. Comparison of Inhibitory Effect of Polyclonal Antisera from Different Rabbits Produced with PEAA in Indirect ELISA

	B/B_0[a]		
	antisera from different rabbits		
concentration of PEAA (μg/L)	R_1	R_2	R_3
0	100	100	100
0.1	82	89	80
1	53	75	46
10	24	56	13

[a]Results are presented as the ratio B/B_0, where B is the mean absorbance of wells in the presence of a given concentration of PEAA and B_0 is the mean absorbance of wells in the absence of PEAA.

Analytical Performance of the Direct ELISA Kit. The effects of ionic strength and pH of the dilution buffer were studied. With an increase of salt concentration, the absorbance decreased (data not shown). Thus, 10 mmol/L PBS was chosen as the optimal dilution buffer. As regards the buffer's pH value, considering that acidic and alkaline solutions may cause changes in the spatial structures or denaturation of the antibody and/or enzyme conjugate, pH 7.0 was selected for further studies.

A 10-point representative inhibition curve obtained using the direct ELISA is presented in Figure 4A. The limit of detection

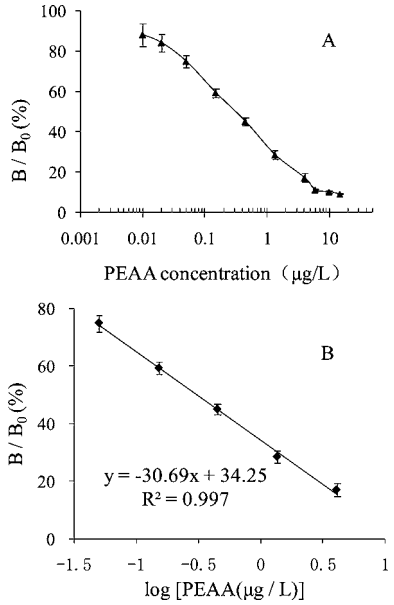

Figure 4. (A) Representative inhibition curve and (B) standard calibration curve for PEAA in direct ELISA. Each point represents the average of five replicates. The concentrations of PEAA standard solution in (A) are 0.01, 0.02, 0.05, 0.15, 0.45, 1.35, 4.05, 6.00, 10.00, and 15.00 μg/L, respectively.

(LOD, also called the least detectable dose) that estimated as the concentration of PEAA giving a 15% inhibition of the maximum absorbance was about 0.02 μg/L. As compared to the previous reports on the detection of other β-agonists compounds, such as detecting clenbuterol using chromatography/electrospray ion trap multiple-stage mass spectrometry and detecting ractopamine residues using enzyme-linked immunosorbent assay,[20,21] the LOD for PEAA in this assay is at the similar level. The average IC_{50} is 0.3 μg/L, and the linear working range is from 0.05 to 4.05 μg/L (Figure 4B). It indicates that this assay is promising in the detection of PEAA residues because of high sensitivity.

Specificity. The specificity of the antibody in optimized assays was evaluated by the measurement of cross-reactivity using PEAA and 15 related compounds as described. The cross-reactivity studies were carried out by the direct competitive ELISA by adding various free competitors at different concentrations to estimate their respective IC_{50} value and then comparing this value with that of PEAA. Table 3 presents the results as a percentage of cross-reactivity with respect to

Table 3. Cross-Reactivities among PEAA and Other β-Agonist Compounds in Direct ELISA

competitor	cross-reactivity[a] (%)
PEAA	100
clenbuterol, terbutalin, cimaterol, fenoterol, tulobuterol, salbutamol, penbutolol, clorprenaline, brombuterol, mabuterol, zilpaterol, cimbuterol, bambuterol, formoterol	<0.1
ractopamine	0.3

[a]Cross-reactivity (%) = ($IC_{50,\ PEAA}/IC_{50,\ competitor}$) × 100%.

phenylethanolamine A. The data demonstrated that the antibody was highly specific for PEAA, with negligible cross-reactivity with ractopamine (CR is 0.3%) and other β-agonists compounds (CR < 0.1%). It is an advantage that the polyclonal antibodies are specific for PEAA and do not cross-react with other β-agonists. In this case, the developed ELISA would be more helpful to monitor the newly emerged alternative PEAA. In fact, PEAA and ractopamine are structurally similar. As Figure 1 shows, the $-OCH_3$ group and $-NO_2$ group on the two benzene rings of PEAA molecule are the structural differences between them. Because the $-NO_2$ group of PEAA molecule was reduced to $-NH_2$ group as the binding site in the synthesis of immunogen PEAA–HSA, the main antigenic determinant for the developed antibodies should be the $-OCH_3$ group in this research. This −OH group on the benzene ring of ractopamine molecule makes significant decrease for the affinity of ractopamine toward the anti-PEAA antibody, further indicating that the $-OCH_3$ group in the PEAA system is the main antigenic determinant.

Inter- and Intra-assay Variation Determination. It is known that various substances existing in complex matrixes can affect the interaction between antigen and antibody in immunoassays. To reduce matrix effects, two common methods could be used, including cleaning the sample and dilution of the sample, which are time-consuming and laborious. Precision of this assay was assessed by measurements of known urine samples without any pretreatment or dilution of the sample. The blank urine samples were spiked with a final PEAA concentration of 0.5, 1.0, and 1.5 μg/L. The obtained mean values ± SD and CV by replicate analyses in the same (intra-assay) and separate (inter-assay) runs are reported in Table 4.

Table 4. Intra- and Inter-assay Variations of Urine Sample Spiked with PEAA

fortified concentration (μg/L)	replicates	mean[a] ± SD (μg/L)	recovery (%)	CV[b] (%)
		Intra-assay[c]		
0.5	6	0.55 ± 0.10	110	17.5
1.0	6	0.84 ± 0.08	84	8.9
1.5	6	1.21 ± 0.06	81	4.8
		Inter-assay[d]		
0.5	6	0.52 ± 0.10	103	19.1
1.0	6	0.98 ± 0.13	98	13.4
1.5	6	1.48 ± 0.28	99	19.1

[a]Concentration of PEAA measured. [b]Coefficient of variation. [c]Intra-assay variation was determined by six replicates on a single day. [d]Inter-assay variation was determined by six replicates on three days.

The urine sample recovery rates were determined from a standard curve with PEAA concentrations of 0, 0.05, 0.15, 0.45, 1.35, and 4.05 μg/L. The recovery rates ranged from 81% to 110%, which indicated relatively good parallelism and accuracy of the assay when applied to real samples. The coefficient of variation was below 18% for intra-assay and below 20% for inter-assay, demonstrating an acceptable level of precision.

In addition, the sensitivity of this assay for real samples was also evaluated by examining 20 blank urine samples. After calculating, the detection limit (mean value +3SD) of this assay for real urine samples was 0.5 μg/L, which is comparable with that of detection of PEAA using the liquid chromatography tandem mass spectrometry method, [14] and is also comparable with that of using immunochemical approaches to detect

ractopamine residues in edible animal products[20] and a variety of β-agonists in plasma samples,[12] and is superior to that of detecting a variety of β-agonists in feed samples.[12]

Comparison of ELISA and UPLC−MS/MS Methods. The collected four urine samples were analyzed using ELISA and UPLC−MS/MS methods. As Table 5 shows, comparing S_2 with other urine samples, individual differences were observed between these four pigs, which may have resulted from the differences of the feed intake. All four urine samples are tested strong positive (no false negatives) by the developed ELISA in the analysis of real samples, which is largely consistent with that detected by the UPLC−MS/MS method. Some differences were also observed between the results by these two methods, which may have resulted from too many dilution times (50 times) before detection of the high concentration of PEAA in urine samples using ELISA method. Considering that PEAA is a kind of prohibited drug in China, the developed ELISA method is relatively reliable for the detection of PEAA residues in urine samples. From a practical point of view, the prototype kit could be advantageously used for the rapid screening of large groups of urine samples, and the kit employed has reliability even in routine application for the control of the illegal use of the drug.

Table 5. Comparisons of PEAA Assay between the ELISA and UPLC−MS/MS Methods in Pig Urine Samples

urine samples	ELISA (μg/L)	UPLC/MS/MS (μg/L)
S_1	13.0	13.4
S_2	319.4	289.3
S_3	33.9	12.8
S_4	17.2	10.6

In summary, we developed a direct competitive ELISA method for the detection of PEAA residues. As compared to the commercially available PEAA ELISA test kit (Shenzhen Lvshiyuan Biotechnology Co., Ltd., Shenzhen, China), the antibody in the newly developed ELISA shows higher sensitivity, because the IC_{50} is several times lower, and the linear working range starts from lower concentration. When applied to pig urine samples, the developed ELISA shows high sensitivity and reproducibility, and the parallelism and accuracy are as good as the commercial kit, and the LOD is comparable. In addition, the direct format needs fewer assay steps and then is more rapid and convenient for screening of large groups of samples than is the commercial kit, which is based on an indirect ELISA. The developed assay exhibits excellent specificity for the PEAA measurements, because the antibody shows negligible cross-reactivity with other β-agonists compounds. Largely consistent results obtained by ELISA and UPLC−MS/MS methods confirmed its reliability for applications in the rapid screening of PEAA in urine samples. From a practical point of view, the kit has reliability even in routine application for the control of the illegal use of the drug.

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Notes

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ABBREVIATIONS USED

PEAA, phenylethanolamine A; β-agonists, β-adrenergic agonists; ELISA, enzyme-linked immunosorbent assay; CR, cross-reactivity; HPLC, high-performance liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; GC−MS, gas chromatography−mass spectrometry; HPLC−MS/MS, high-performance liquid chromatography tandem mass spectrometry; HRP, horseradish peroxidase; OVA, ovalbumin; HSA, human serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine; SAS, saturated ammonium sulfate; OD, optical density; IC_{50}, 50% inhibitory concentration; LOD, the limit of detection; SD, standard deviation; CV, coefficient of variation

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