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Generation of anti-trenbolone monoclonal antibody and establishment of an indirect competitive enzyme-linked immunosorbent assay for detection of trenbolone in animal tissues, feed and urine

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ABSTRACT

Trenbolone (TRE) is a steroid used by veterinarians on livestock to increase appetite and body weight. The use of TRE has been restricted because of its harmful side effect for consumers. To effectively control TRE residue in food and food product, a rapid and convenient immunoassay was developed by preparing an anti-TRE monoclonal antibody. The immunogen and coating antigen were prepared by coupling TRE hapten with carrier proteins via 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) method. The optimized method gave an average IC_{50} value of 0.323 ng mL^{-1} towards TRE and an average detection limit (LOD) of 0.06 ng mL^{-1}, which is much lower than the maximum residue levels (2.0 ng g^{-1}) accepted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The specificity of the antibody was evaluated by measuring cross-reactivity of six structurally related compounds, including 19-nortestosterone (9.7%), testosterone (0.13%), methyltestosterone (<0.01%), methandrostenolone (<0.01%), (+)-dehydroisoandrosterone (<0.001%) and β-estradiol (<0.001%). The recovery rates of the test in detection of TRE-fortified animal tissue, urine and animal feed samples were in the range of 81.3–89.4%, while the intra- and inter-assay coefficients of variation were less than 12.0%.

1. Introduction

Trenbolone (17β-hydroxyestra-4,9,11-trien-3-one, TRE, Fig. 1) is a steroid with progestomimetic and anti-gonadotrophic effects, and shows a weak oestrogenic, anabolic and androgenic efficacy. TRE has been commonly utilized by veterinarians on livestock to increase muscle growth and appetite. As a result, the body weight of the animal increases more effectively [1]. However, the administration of TRE spiked feed would induce several adverse events to the animals and consumers who have taken the animal as food. The side effects include insomnia, high blood pressure and night sweats. Owing to these harmful side effects, the utilization of TRE has been strictly regulated in many countries. Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set MRLs (maximum residue levels) for TRE of 2 ng g^{-1} in muscle and 10 ng g^{-1} in liver in the Report "TRS 788-JECFA 34/40". Also, the Ministry of Agriculture in People's Republic of China issued the document no. 235 which claimed that TRE should not be detectable in the animal-origin food. Hence, to monitor TRE residue in animal feeds and tissues, a convenient, rapid and sensitive screening method is urgently required.

The present methods to detect TRE are instrumental methods, including high-performance liquid chromatography (HPLC) [2], gas chromatography–mass spectrometry (GC–MS) [3], liquid chromatography (LC) [4] and LC–MS [5–8]. These methods are accurate, but the instruments are expensive and not fit for rapid screening analysis. On the contrary, enzyme linked immunosorbent assay (ELISA) is well-accepted, low-cost, rapid, high-capacity and sensitive, and has been more and more applied in detection of drug residues [9–12]. Fitzpatrick et al. [9] prepared polyclonal antibodies to TRE and developed ELISA method to detect TRE in bovine bile, with the detection limit (LOD) of 3 ng mL^{-1}. Meyer and Hoffman [10] and Degand et al. [11] also produced specific anti-

Abbreviations: BSA, bovine serum albumin; cFA, complete Freund's adjuvant; DMF, *N*,*N*-dimethyl formamide; EDC, 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride; ELISA, enzyme linked immunosorbent assay; IC_{50}, concentration at 50% inhibition; iFA, incomplete Freund's adjuvant; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; LOD, limit of detection; MRL, maximum residue level; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine; TRE, trenbolone.

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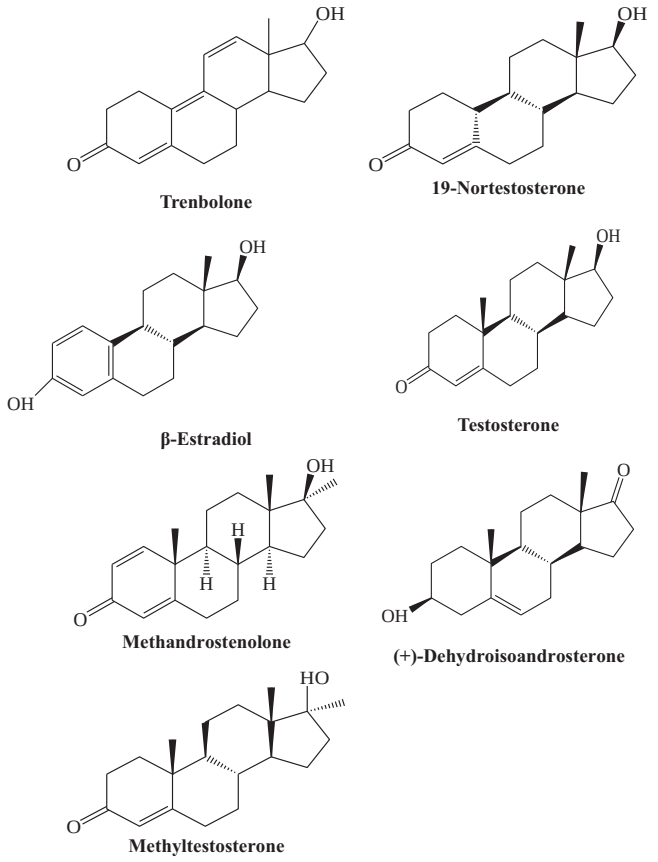

Fig. 1. Structures of trenbolone (TRE) and related compounds evaluated in this study.

bodies for TRE in rabbits, using TRE–bovine serum albumin (BSA) as the immunogen, and detected TRE in biological samples with the TRE–alkaline phosphatase (AP) and TRE–horseradish peroxidase (HRP) as the tracer, respectively.

Considering the advantages of monoclonal antibody (MAbs) in high sensitivity and specificity, we aimed to produce the MAbs for TRE and develop a more sensitive ELISA method for TRE detection in different matrices, including animal feed, tissue and urine. To our knowledge, the generation of the MAbs for TRE has not been reported yet.

2. Experimental

2.1. Chemicals and materials

Trenbolone (TRE), 19-nortestosterone, testosterone, methandrostenolone, (+)-dehydroisoandrosterone, methyltestosterone and β-estradiol were purchased from J&K Scientific Ltd. (Beijing, China). 1-Ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), Freund's complete adjuvant (cFA) and Freund's incomplete adjuvant (iFA) were from Sigma–Aldrich (St. Louis, MO). Goat anti-mouse IgG-horseradish peroxidase (HRP), succinic anhydride and *N*-hydroxysuccinimide (NHS) were from Military Medical Institute (Beijing, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Shanghai Sangon Biological Engineering Technology (Shanghai, China). Dimethylformamide (DMF), methanol, hydrogen peroxide (30%) and other reagents were of chemical grade and supplied by Guangmang Chemical Co. (Jinan, China).

2.2. Instrumentations

ELISA analysis was performed in polystyrene 96-well microtiter plates (Bio Basic Inc.) and spectrophotometrically read with an automatic microplate reader KHB ST-360 from Shanghai Zhihua Medical Instrument Ltd. (Shanghai, China). Protein dialyses were

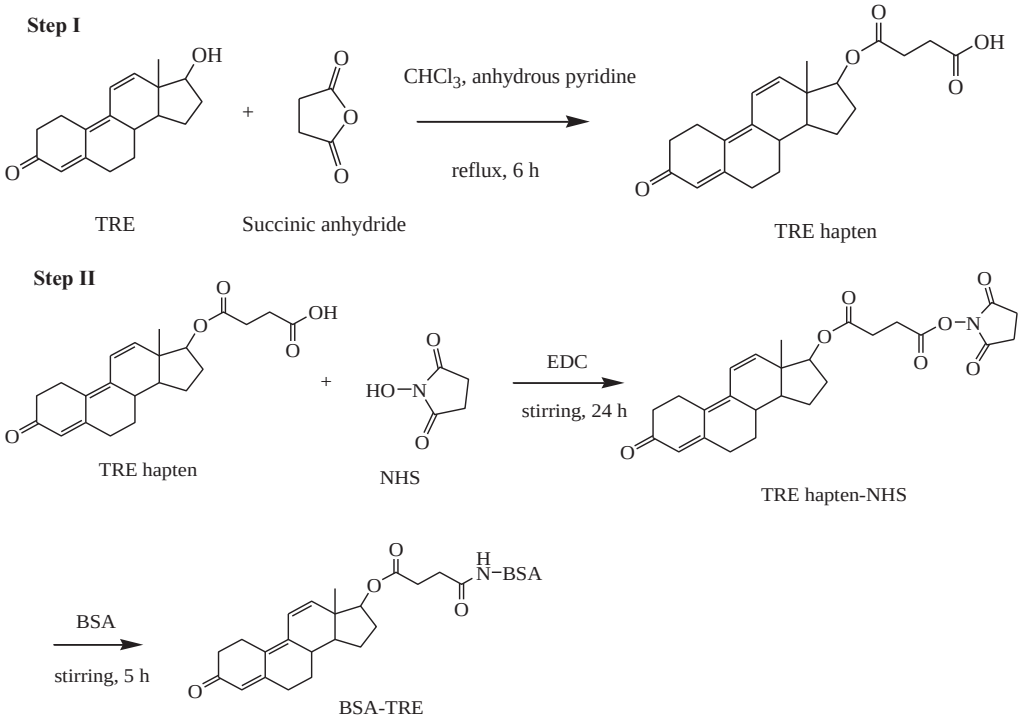

Fig. 2. Preparation of TRE hapten (Step I) and coating antigen (BSA–TRE conjugate, Step II).

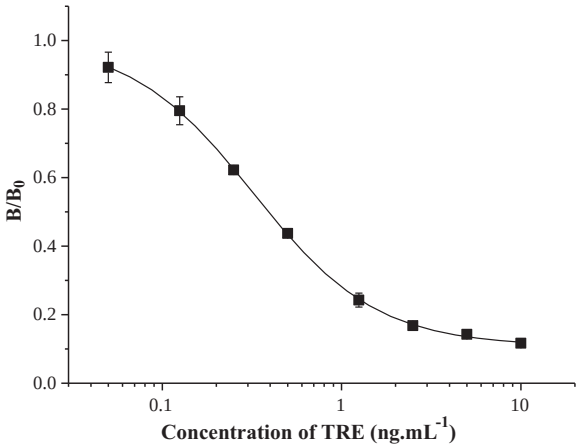

Fig. 3. Representative inhibition curve of ELISA analysis using TRE as the competitor. Each point represents the average value of five replicates and the IC_{50} value was found to be 0.323 ng mL^{-1}.

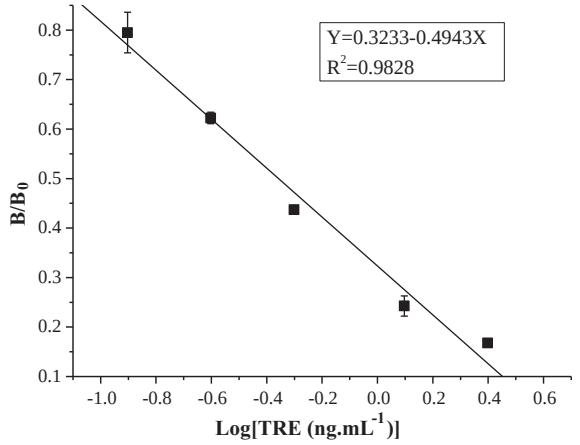

Fig. 4. Standard calibration curve of ELISA analysis with TRE as the competitor for binding anti-TRE antibody with coating antigen.

performed using dialysis tubes from Aibo Economic & Trade Co., Ltd. (Jinan, China). Centrifugation was carried out with a refrigerated centrifuge (Biofuge Stratos, Heraeus).

2.3. Buffers

For preparation of all buffers for the immunoassay, ultrapure deionized water was used. Phosphate-buffered saline (PBS, pH 7.4) was composed of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4 and 2.7 mM KCl. The wash buffer (PBST) was a PBS buffer containing 0.05% Tween 20 and 1‰ sodium azide (NaN_3). 0.05 M Na_2HPO_4–citric acid buffer (pH 8.0) was used as the coating buffer. The blocking buffer was PBS + 1% of BSA + 0.05% (v/v) Tween 20. The substrate buffer was 0.1 M sodium acetate/citrate buffer (pH 5.0). The working buffer was 50 mM Tris–HCl (pH8.0). The peroxidase substrate solution was prepared by mixing 200 μL of 1% (w/v) TMB in DMSO and adding 64 μL of 0.75% (w/v) to 20 mL of substrate buffer. The enzymatic reaction was stopped with 2 M H_2SO_4.

Table 1
Intra-assay and inter-assay variation for TRE standard solution (0.45 ng mL^{-1}) tested by the ELISA method.

Detection number	Coefficient of variation (CV, %)		
	Day 1	Day 2	Day 3
1	5.4	6.1	5.7
2	8.1	7.4	4.1
3	4.7	8.1	6.2
4	7.4	9.5	8.7
5	6.1	6.3	7.2
6	8.2	4.8	5.6
7	8.4	7.1	4.8
8	7.4	9.2	5.6
9	8.6	5.4	9.2
10	9.1	6.1	7.3
Intra-assay variation (%)	7.3	7.0	6.4
Inter-assay variation (%)		6.9	

Table 2
IC_{50} and percentage of cross-reactivity of selected competitors.

Competitor	IC_{50} (ng mL^{-1})	Cross reactivity[a] (%)
Trenbolone	0.323	100
19-Nortestosterone	3.326	9.7
Testosterone	244.9	0.13
Methyltestosterone	>1000	<0.01
Methandrostenolone	>1000	<0.01
(+)-Dehydroisoandrosterone	>10,000	<0.001
β-Estradiol	>10,000	<0.001

[a] The percentage of cross-reactivity is defined as the ratio of IC_{50} value for TRE to that for competitors.

2.4. Preparation of TRE hapten

The hapten of TRE was synthesized by succinic anhydride method (Fig. 2, Step I) [13,14]. In brief, 0.27 g TRE and 0.4 g succinic anhydride was mixed in a 50 mL of flask, followed by adding 4 mL of chloroform and 1 mL of anhydrous pyridine. Then the mixture was refluxed for 6 h. After that, the reaction mixture was purified by column separation to get TRE hapten using $CHCl_3$:*n*-hexane:methanol (5:5:1, v/v/v) as elute solution.

2.5. Preparation of coating antigen and immunogen of TRE

The coating antigen of TRE was prepared via an EDC/NHS reaction [15]. As is indicated in Fig. 2 (Step II), 10 mg TRE hapten, 30 mg EDC and 15 mg NHS were added to 1 mL of DMF and the mixture was stirred for 24 h to get solution I. Then 61 mg BSA in 3.0 mL of PBS (solution II) was dissolved and added to the solution I (hapten:BSA = 30:1, molar ratio). The mixture was stirred at room temperature for 5 h. After that, the reaction mixture was purified by dialyzing (mwco, 12,000–14,000 Da) against PBS (0.01 M, pH 7.4) for three days to remove the uncoupled hapten. Finally, the mixture was centrifuged at 12,000 rpm for 30 min. The supernatant was then collected to get coating antigen BSA–TRE. The immunogen KLH–TRE was synthesized by the same procedure, except that 25 mg KLH was used in the preparation of solution II.

2.6. Generation and purification of anti-TRE monoclonal antibody (MAbs)

BALB/c mice were subcutaneously injected with 100 μg immunogen formulated in cFA. Later, mice were immunized for totally four times at two-week intervals with immunogen in iFA. Intraperitoneal injection was carried out after the fourth injection. Titer antiserum was determined via tail bleeding before the animals were scarified for spleen collection. Hybridomas were taken [16,17] and the isolated splenocytes were fused with the murine myeloma SP/20 in the ratio of 5:1. Culture supernatants from each clone were subjected to screening by indirect ELISA as described below. Positive ones were subcloned by limiting dilution. In order to gain massive monoclonal antibodies, mice were injected intraperitoneally with 0.5 mL of antiseptic paraffin oil. Seven days later, 5×10^6 hybridomas were inoculated into abdominal cavity for each

Table 3
Effect of matrix on the accuracy result for TRE determination by the ELISA method.

Sample	Animal tissue		Urine		Animal feed	
Spiked level	1.0 ng g^{-1}	2.5 ng g^{-1}	1.0 ng g^{-1}	2.5 ng g^{-1}	1.0 ng g^{-1}	2.5 ng g^{-1}
Recovery (%)						
1	82.4	76.4	84.3	91.4	83.4	87.4
2	87.4	83.6	82.7	92.7	86.7	82.7
3	79.8	84.6	81.6	89.7	84.6	84.6
4	84.1	83.2	78.4	83.9	94.2	76.1
Mean (%)	83.4	82.0	81.3	89.4	87.2	82.7
CV (%)	3.8	4.6	3.1	4.3	5.6	5.8

mouse. One week later, 6.0 mL of ascites was collected. The antibody was purified via ammonium sulfate precipitation method [18] and stored at −20 °C for use.

2.7. Establishment of indirect competitive ELISA method

To optimize the coating antigen and the antibody concentrations, the check board procedure was carried out as follows. The 96-well plate was coated with 100 μL of coating antigen (BSA–TRE) for 2 h at 37 °C and overnight at 4 °C, then washed for four times. The excess binding sites were blocked with 250 μL/well of blocking buffer, followed by incubation for 2 h at 37 °C. After the plate was washed as described above, anti-TRE antibody was added (50 μL/well), followed by addition of buffer with or without competitor (TRE or other competitors, 50 μL/well), and the plate was incubated for 0.5 h. After washing, goat anti-mouse IgG HRP (100 μL/well) was added, followed by incubation for 0.5 h at 37 °C. Unbound goat anti-mouse IgG HRP was removed by washing and TMB substrate solution was added (100 μL/well). After the plates were incubated for another 15 min at 37 °C, the chromogenic reaction was inhibited by adding stopping solution (100 μL/well), and absorbances were measured at 450 nm. Absorbances were corrected by blank reading with preimmune serum used as a negative control. The result was expressed as percentage inhibition (B/B_0), where B is the absorbance of the well containing analyte and B_0 is the absorbance of the well without analyte [15,19]. Inhibition curve was plotted as B/B_0 versus logarithm of TRE concentration (0, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5.0 and 10 ng mL^{-1} in PBS) [20].

2.8. Antibody specificity determination

Cross reactivity (CR) was tested to determine the specificity of the antibody. Six structurally related compounds [19-nortestosterone, testosterone, methandrostenolone, (+)-dehydroisoandrosterone, methyltestosterone and β-estradiol, Fig. 1] were selected and evaluated. Standard solutions of testing compounds were analyzed by the ELISA procedures described above. CR was expressed as the ratio of IC_{50} values for TRE to that for competitors, e.g. CR (%) = ($IC_{50\ trenbolone}/IC_{50\ competitor}$) × 100% [21].

2.9. Matrix effect evaluation

To evaluate the matrix effect on the accuracy and precision of the method, animal feed, tissue and urine spiked with TRE were analyzed. The tested matrices were firstly treated as follows. Feed or animal tissue: Feed or animal tissue samples (5 g) were homogenized and shaken for 10 min in the mixture containing 8 mL of

Table 4
Inter- and intra-assay variation for raw matrix spiked with 2.5 ng g^{-1} TRE.

Sample	Day	Sample number	Measured (ng g^{-1})					Mean (ng g^{-1})	Recovery (%)	Intra-assay CV (%)	Inter-assay CV (%)[a]
Animal tissue	1	A	2	1.6	2	2.2	2.1	1.9	79.2	11.5	**10.1**
		B	1.8	2	2.1	2.3	2.2	2.1	83.2	9.3	
		C	2	2.1	1.9	1.8	2	2.0	78.4	5.8	
	2	A	2.3	2.2	2	1.8	1.7	2	80.0	12.8	
		B	2	1.8	2.3	1.7	2.2	2	80.0	12.8	
		C	1.8	2.3	1.7	1.9	2	1.9	77.6	11.9	
	3	A	2	1.8	1.9	2	2.3	2	80.0	9.4	
		B	2.4	2.2	2.1	1.9	2.3	2.2	87.2	8.8	
		C	1.8	1.9	2.1	2.2	1.9	2.0	79.2	8.3	
Urine	1	A	2.1	1.8	2.4	2.4	2.3	2.2	88.0	11.6	**11.7**
		B	2	1.9	2.3	1.8	2.1	2.0	80.8	9.5	
		C	1.7	1.8	2.2	1.9	2.3	2.0	79.2	13.1	
	2	A	1.8	1.9	2.1	1.8	2.2	1.9	78.4	9.3	
		B	2	2.3	2.2	1.8	1.7	2.0	80.0	12.8	
		C	1.8	2.1	2	2.3	1.9	2.0	80.8	9.5	
	3	A	1.9	2.1	1.7	2.3	2.2	2.0	81.6	11.8	
		B	2.2	1.8	1.7	1.9	2.5	2.0	80.8	16.2	
		C	1.8	2.4	1.9	2.1	2.1	2.1	82.4	11.2	
Animal feed	1	A	1.8	2	2.1	1.9	2.3	1.8	80.8	9.5	**10.1**
		B	2.5	2.3	2.1	2.2	1.9	2.5	88.0	10.2	
		C	2.3	2.1	2.2	1.7	2	2.3	82.4	11.2	
	2	A	1.9	2.1	1.8	2.3	2	1.9	80.8	9.5	
		B	2.1	2.3	2.2	1.8	1.9	2.1	82.4	10.1	
		C	2.2	1.9	2.1	2.4	2	2.2	84.8	9.1	
	3	A	2.3	2.2	2.1	1.8	1.9	2.3	82.4	10.1	
		B	1.9	1.9	2.2	2	2.4	1.9	83.2	10.4	
		C	2.1	2.2	1.8	1.7	1.9	2.1	77.6	10.7	

[a] Inter-assay CV was determined three replicates on three different days (n = 3).

acetonitrile and 2 mL of 1 M NaOH solution. The mixture was centrifuged at 3000 rpm for 10 min at room temperature. Then 1 mL of supernatant was mixed with 1 mL of 1 M NaOH and 2 mL of acetonitrile. After vortexing for 1 min, 2.4 mL of *n*-hexane and 1.6 mL of dichloromethane were added. Then the mixture was shaken for 10 min and centrifuged for 10 min at 3000 rpm at room temperature. The upper *n*-hexane layer was removed and evaporated to dryness in the nitrogen flow. The residue was dissolved in 1 mL of working buffer and then diluted 4 times with PBS. Then 50 μL of this solution was used for analysis.

Urine: 2 mL of urine was centrifuged at room temperature for 10 min at 3000 rpm. Then 1 mL of the supernatant was mixed with 2 mL of acetonitrile and 3 mL of *n*-hexane/dichloromethane (4:1, v/v). After vortexing for 10 min, the mixture was centrifuged at 3000 rpm for 10 min at room temperature. The upper *n*-hexane layer was removed and evaporated to dryness in the nitrogen flow. The residue was dissolved in 1 mL of the working buffer and used for analysis.

To evaluate the accuracy of the test, recovery rates were determined. Animal tissue, urine and animal feed samples were fortified by TRE at the final concentrations of 1.0 and 2.5 ng g^{-1}, according to the maximum residue level accepted by FAO/WHO (2.0 ng g^{-1}). The spiked samples were treated as the procedures described above and analyzed by the method to get the measured level in each sample from the standard calibration curve. The recovery results were calculated as the ratio of measured level to spiked level ($n = 4$).

To evaluate the precision of assay, inter-assay and intra-assay variation were determined. Three batches of animal tissue, urine and animal feed samples were spiked with TRE at the final concentration of 2.5 ng g^{-1}, and then detected by the ELISA method to measure the TRE level in each sample on three different days and calculate the recovery and coefficient of variation (CV) for each group. Inter-assay variation was determined three replicates on three different days ($n = 3$); intra-assay variation was determined five replicates on a single day ($n = 5$).

3. Results and discussion

3.1. Synthesis of coating antigen and immunogen

To synthesize an immunogen, the analyzed hapten must bear a spacer (carboxyl, amino, or hydroxyl group) to couple with the carrier protein. Both the spacer length and connection position at the hapten molecule are important in the production of a successful antibody. Generally, the molecular structure of the original compound should not be changed too much. A spacer length of 3–5 C-atoms is considered to be the most suitable [21]. Considering the active hydroxide group in the structure of TRE (Fig. 1), succinic anhydride method was used in this study and the desired hapten was acquired (Fig. 2). The TRE hapten bearing a 4 C-atoms carboxylic acid reactive group was synthesized and then covalently coupled with the amino group in the carrier protein (NH_2–KLH and NH_2–BSA) to get immunogen and coating antigen, respectively [22].

3.2. Optimization of the ELISA method

Two parameters were evaluated to optimize the ELISA method, including IC_{50} (the concentration of standard solution producing 50% inhibition) and limit of detection (LOD). To establish a highly sensitive and specific ELISA method, the optimum assay conditions were determined by adjustment of several parameters, such as the concentration of coating antigen, dilution of antiserum and goat anti-mouse IgG-HRP [23]. According to the result of checkerboard titration assay, the optimized concentration of coating antigen BSA–TRE was found to be 30 ng mL^{-1}. The dilution for MAbs was 1:14,000 and the suitable dilution of goat anti-mouse IgG-HRP was 1:2000.

Under the selected conditions, the representative ELISA inhibition curve for TRE detection was constructed at the final TRE concentrations of 0, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5.0 and 10 ng mL^{-1} (Fig. 3). The sensitivity was expressed as IC_{50} value. The lower of the IC_{50} value was, the higher sensitivity of the assay would be. In this study, the average value of IC_{50} was 0.323 ng mL^{-1} (Fig. 3), which is much lower than the maximum residue levels issued (2.0 ng g^{-1}) by FAO/WHO, indicating the satisfactory sensitivity of the antibody. Besides, the LOD value was considered to be the concentration giving a 10% inhibition of the maximal absorbance value [24] and calculated to be 0.06 ng mL^{-1}. The linear working range was 0.125–2.5 ng mL^{-1} (Fig. 4). Compared with the Fitzpatrick' work [9], in which TRE was conjugated with thyroglobulin to immunize rabbit (LOD = 3 ng mL^{-1}), the MAbs in our study was about 50 times more sensitive (LOD = 0.06 ng mL^{-1}). All these results demonstrated the excellent sensitivity of the assay.

To test the stability of the method, 0.45 ng mL^{-1} TRE standard solution was evaluated ten times on a single day to get intra-assay variation, or over three different days to get inter-assay variation. As is shown in Table 1, both intra- and inter-assay variation were less than 8.0%, suggesting the acceptable repeatability of the method.

3.3. Specificity of the MAbs

The specificity of the antibody was evaluated by measuring inhibition curves using six structurally related compounds as the competitors, including 19-nortestosterone, testosterone, methyltestosterone, methandrostenolone, (+)-dehydroisoandrosterone and β-estradiol (Fig. 1). Cross-reactivity was measured by comparison of the IC_{50} of the TRE with that of competitors (Table 2). These results demonstrated that only 19-nortestosterone showed a little cross-reactivity (9.7%) with the antibody. This observation could be probably explained by the fact that 19-nortestosterone exhibited the most similar structure to TRE among the six compounds.

3.4. Matrix effect evaluation

For the accuracy test, animal tissue, urine and feed samples were spiked with 1.0 ng g^{-1} and 2.5 ng g^{-1} of TRE. Unspiked samples were served as the blank groups. The extracts were diluted with PBS to minimize the matrix effect. Accuracy was tested by detection four replicates and the results are shown in Table 3. For the two spiked levels, the recovery rates were all greater than 81.3% for the three kinds of matrices and the coefficient variations were 3.1–5.8%. To evaluate the precision of the method, three batches of the matrices were fortified with TRE at the concentration of 2.5 ng g^{-1} and detected by the similar procedure five replicates on a single day, and continued for three days. As is shown in Table 4, the recovery rates were all more than 77.6% of theoretical values and the coefficient variations were in the range of 10.1–11.7% for inter-assay ($n = 3$) and 5.8–16.2% for intra-assay ($n = 5$) variation in three samples, indicating the satisfactory precision of the method in the presence of selected matrices. These results demonstrated that the method established has the potential to be used for rapid detection of TRE in actual samples.

4. Conclusion

In this research, hapten derivative with a four-carbon-atom length of carboxylic spacer was designed and synthesized to produce monoclonal antibody to TRE. An indirect competitive ELISA method was thus developed and optimized for detection of TRE in spiked feed, animal tissue and urine samples. The antibody showed excellent sensitivity towards TRE with an average IC_{50}

value of 0.323 ng mL^{-1} and LOD of 0.06 ng mL^{-1}. The linear range for standard calibration curve was from 0.125 to 2.5 ng mL^{-1}. The cross-reactivity results displayed that the antibody was highly specific to TRE in six structurally and functionally related compounds. The average recovery rates in animal feed, tissue and urine system were from 81.3 to 89.4%, and coefficient variation was less than 6.0% for the spiked level of 1.0 and 2.5 ng g^{-1}. The inter-assay variation was 10.1–11.7% and intra-assay was 5.8–16.2%. In a word, this ELISA test could be hopefully used as an alternative screening method to detect TRE in animal feed, tissue and urine samples.

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