Letter to the Editor

Screening Testosterone Residue in the Edible Tissues of Bovine with a Polyclonal Antibody Based Heterologous Immunoassay*

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Testosterone (17β-hydroxyandrost-4-en-3-one) is a 19-carbon steroid hormone with potent androgenic properties. It maintains testicular function and develop secondary male sex characteristics. It also has strong anabolic effects, which initiates increased protein synthesis in muscle and bone[1]. In the 1950s, the recognition of the growth promoting properties of such a hormone led to its introduction as a tool to increase meat production[2]. Concern over the use of hormone implants first surfaced during the late 1970s and early 1980s, when a number of incidents linked with hormone residues in meat with various conditions in children, such as premature sexual development, and ovarian cysts in adult women (e.g. uterine and ovarian cancers)[3]. This prompted the EU to introduce a number of directives culminating in a ban on the use of growth promoting hormones within the EU, except for therapeutic or other veterinary purposes.

The majority of methods available to estimate testosterone excretion are based on high-performance liquid chromatography (HPLC)[4], gas chromatography coupled with mass spectrometric (GC-MS)[5], and liquid chromatography coupled with mass spectrometric (LC-MS)[6], that involve multi-step procedure such as hydrolysis, extraction, derivative formation, and purification. Contrary to these traditional chromatographic methods, several immunoassay methods are portable and are proved to be cost-effective, with adequate sensitivity, high selectivity, and simple sample extraction process for the detection of testosterone residues in biological matrices[7]. However, there is few immunoassays published for testosterone detection with respect to bovine edible tissues. It was for this reason we aimed in the present study to produce polyclonal antibody (pAb) and develop a sensitive indirect competitive ELISA for the detection of testosterone residue in bovine muscles. Following the minimization of sample pre-treatment, we also discussed the organic solvents tolerance and matrix effects.

Therefore, two different artificial antigen synthesis procedures were employed in our study to expect the heterologous sensitivity and the feasibility to apply this polyclonal antibody in a competitive ELISA to detect TES residues. Firstly, EDC method was employed to synthesize the immunogen of TES-17-BSA, and mixed-anhydride technique was employed to synthesis the TES-3-OVA coating antigen. Then, TES-17-BSA was used to raise anti-TES polyclonal antibodies using two female New Zealand white rabbits, and antibody titers were tested by indirect ELISA as described before[8]. Indirect competitive ELISA (icELISA) was employed to determine the sensitivity and specificity of the method. The inhibition rate was expressed as $\%B/B_0$, where B is the absorbance of the well containing competitor and B_0 is the absorbance of the well without competitor. Specificity is defined as the ability of structurally related chemicals to bind to the anti-TES polyclonal antibody, and the cross-reactivities (CR) are calculated as (IC_{50} of TES)/ (IC_{50} of competitors) ×100.

Immunoassay performance may be affected by chemical parameters such as ionic strength, pH values, organic solvent concentration, and sample matrices. The effects of these parameters were estimated by running standard curves under various conditions. The maximum absorbance (A_{max}, the absorbance value at zero concentration of analyte) and IC_{50} value were calculated, and the maximal A_{max}/IC_{50} ratio was chosen. In our study, acetonitrile and methanol were often added to the assay buffer to improve solubility of the analyte. In order to evaluate the effects of these two detergents, TES was diluted in assay buffer with 2%, 5%, 10%, 20%, or 30% acetonitrile respectively, or with varying methanol concentrations (10%, 20%, 30%, 40%, or 50%).

Fresh Jiaxian bovine meat samples that had not been exposed to steroids according to grower logs

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were purchased in retail outlets in Xinxiang, China. After fat and connective tissues were removed by dissection, the samples were homogenized with a high-speed triturator, and were collected in a 50-mL round-bottom plastic flask. Two gram of homogenate was mixed with 2 mL acetonitrile and was centrifuged at 5000 g for 10 min, and then another 2 mL of acetonitrile was added to precipitate to repeat the above procedure. In order to evaluate the matrix effects, supernatants were diluted in PBS (totally 1: 2, 5, 10, and 20 dilution) before they were applied to the microtiter plate. B_0 and IC_{50} values from each diluted curve were compared with that generated from the PBS buffer to determine the appropriate dilution schedule.

During the experiments, the quality of the antisera from two immunized rabbits was tested. From the third immunization, about 0.5 mL of antiserum for each animal was collected via marginal ear vein in 7 days after injection. The titre and affinity of the antisera were primarily determined based on an indirect ELISA procedure. It was found that the titre of the antisera was increased with the times of immunization. After the fifth injection, the titres of two antisera were found to be 1:10 000 and 1:20 000, respectively. Therefore, both rabbits were bled in 10 days after the fifth injection, and the protein contents were determined to be 3.8-4.0 mg/mL after antisera purification. Using icELISA detection, the antiserum with the titer of 1:10 000 showed poor inhibition when TES was used as a competitor; for another antiserum, however, high inhibition was observed. Therefore, the antiserum from the rabbit with the higher titer and inhibition was selected for subsequent ELISA establishment.

It is well known that immunoassay performance may be affected by many physic-chemical features of the media and by a variety of experimental conditions, of which the working concentrations of antibody and coating antigen are crucial factors for the sensitivity of competitive ELISA methods[9]. For this reason, checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the A_{max} was between 1.5-2.0, and the dose-response curve of inhibition ratio versus the TES concentration pursued the lowest IC_{50} values. From the checkerboard assays, the optimal concentration of coating antigen was chosen as 0.8 μg/mL and pAb was 0.2 μg/mL (1:20 000 dilutions). Based on the results of the checkerboard titration, a representative standard curve with icELISA format is shown in Figure 1.

Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of TES that produced 50% inhibition of antiserum binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition[10]. The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20%-80% inhibition rate (IC_{20}-IC_{80} values) of the maximum signal. Therefore, this assay allowed the detection of TES (20%-80% inhibition) from 0.078 to 68.4 ng/mL, with an IC_{50} value of 1.8 ng/mL. The limit of detection (LOD) of the assay was determined to be 0.032 ng/mL.

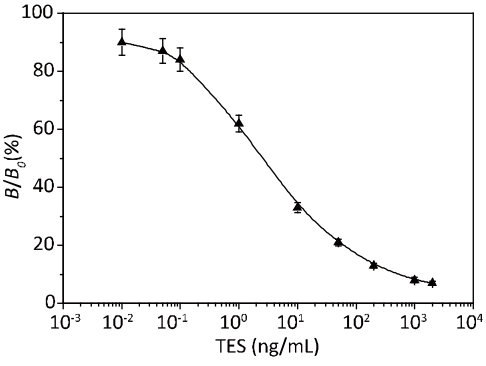

Figure 1. Optimized standard icELISA inhibition curve for TES. Data were obtained by averaging five independent curves, each running in triplicates.

Specificity is a phenomenon inherent to all immunoassays, which is evaluated by determining the cross-reactivity based on the IC_{50} values of individual chemicals. In this work, the study was undertaken by adding various competitors of structural or functional related analogues. Analytes that do not react with the antibody would produce nearly 100% absorbance; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. It can be seen that the established icELISA method demonstrates remarkable specificity toward TES and showed negligible cross-reactivity to other compounds, except for a slight cross-reactivity (19.6%) to 19-nortestosterone.

Acetonitrile and methanol were commonly used to extract TES from samples and increase the solubility of analytes. Their effects on the icELISA were therefore tested. Figure 2 shows the

normalized dose-response curves at various solvent concentrations. First, the effect of acetonitrile from 2% to 30% (v/v) in PBS on the immunosorbent assay was studied (Figure 2A). The rise of the concentration of acetonitrile generally decreased and increased the IC_{50} value afterwards, but a continuous increase in the time for colour development. This is due to that acetonitrile may affect the reaction between antigen and antibody by decreasing the bioactivity of antibody and hindering the enzyme activity. However, as the contents of acetonitrile increased from 2% to 10%, the absorbance gradually approached to that of the PBS buffer, indicating that 10% of acetonitrile still allowed a significant gain in the detectability of this analyte. Taking into account of these findings, acetonitrile concentration in the assay should not be higher than 10%.

Figure 2B shows the normalized dose response curves at different methanol compositions. It was observed that methanol concentrations from 10% to

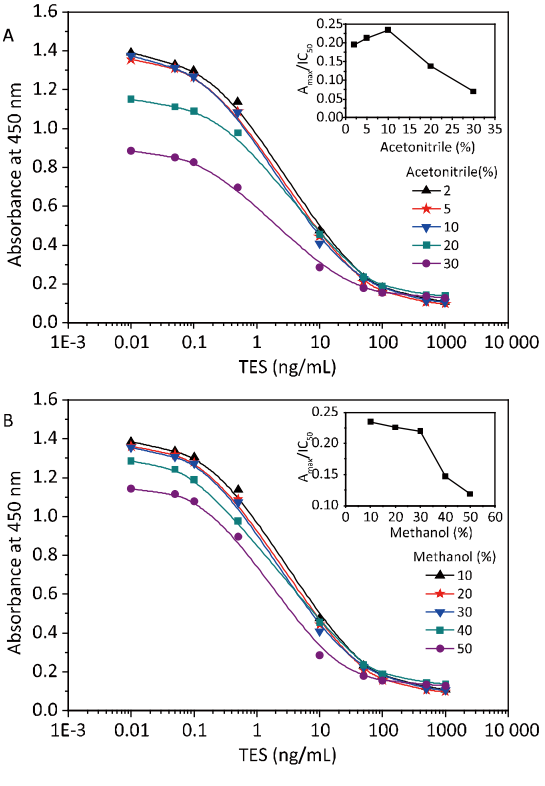

Figure 2. Effects of acetonitrile (A) and methanol concentrations (B) on the icELISA inhibition curve in 0.01 MPBS. Insets indicate the fluctuations of A_{max}/IC_{50} (*Y*-axis) as a function of solvent concentration (*X*-axis). Each value represents the mean of three replicates.

20% had no significant effects on the sensitivity, and the IC_{50} value just changed from 1.8 ng/mL to 1.9 ng/mL. This sensitivity increase may be due to the dispersion and weakening of the nonspecific binding derived from pAbs. Consequently, concentrations of methanol higher than 30% resulted in lower absorbance and sensitivity drop as the higher methanol might have weakened the antibody-hapten interaction. Therefore, it was necessary to control the methanol concentration to be lower than 30% in the sample extract solution. In this study, 20% of methanol in PBS was recommended as the preferred assay buffer, and was employed in the remaining experiments.

It is well known that immunosorbent assays are rapid and convenient for the analysis of food samples because they usually do not require sample pre-concentration and clean-up steps, as compared with traditional chromatographic methods. However, immunoassays often have a high potential for non-specific binding between non-targent analytes and antibodies, known as matrix effects, which may reduce the sensitivity and reliability of the immunoassay and cause false positives by lowering colour development. Chemical substances present in sample or sample extracts, such as solvents, protein, fat and other compounds, may contribute to the matrix effects. Matrix interferences can be reduced in a number of ways, in which dilution with some buffers is a commonly used procedure, and is also employed in this paper.

A comparison between calibration plots for TES prepared in PBS and those prepared in bovine muscles showed clear evidence of matrix effects (Figure 3). As the dilution of bovine muscle extracts

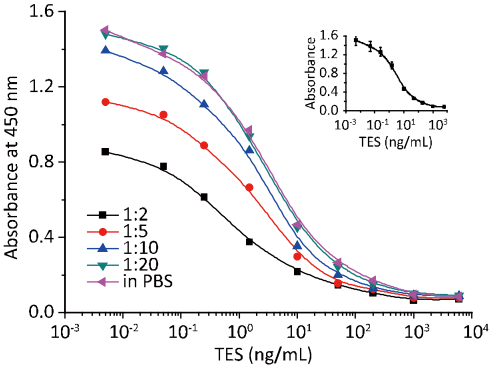

Figure 3. TES standard curves in the diluted bovine muscle samples. Each point represents the average of three separate assays in triplicate. Insets indicate the icELISA standard curve in PBS.

increased from 1:2 to 1:20, the sensitivity was gradually improved to approach the PBS buffer values. The average B_0 (antibody binding with no competitor present) values for dilutions at 1:2, 1:5, 1:10, and 1:20 presented absorbencies of 0.8634, 1.152, 1.397, and 1.569, respectively, as compared to 1.586 in PBS. As Figure 3 shows, the curve of 1:20 dilution had higher goodness-of-fit with standard curve in PBS. Considering the feasibility of screening large amount of samples in a short period of time, bovine sample extracts were determined directly after 1:20 dilution in PBS.

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