ARTICLE

Monoclonal Antibody-Based ELISA and Colloidal Gold Immunoassay for Detecting 19-Nortestosterone Residue in Animal Tissues

Jinqing Jiang,*[†,‡] Ziliang Wang,[†] Haitang Zhang,[†] Xiaojian Zhang,[†] Xingyou Liu,[†] and Sanhu Wang[†]

[†]College of Animal Science, Henan Institute of Science and Technology, Xinxiang, Henan 453003, China
[‡]College of Veterinary Medicine, Northwest A&F University, Yangling, Shanxi 712100, China

ABSTRACT: This article presents the generation of monoclonal antibodies (mAbs) with high specificity against 19-nortestosterone (NT) through cell fusion techniques and the development of a mAb-based indirect competitive ELISA (icELISA) method and colloidal gold-based immuno-chromatographic assay to detect NT residues in beef and pork samples. A modified carbodiimide method was employed to synthesize the artificial antigen, and BALB/c mice were used to produce anti-NT mAbs. On the basis of the checkerboard titration, an indirect competitive ELISA standard curve was established. This assay was sensitive and had a linear range from 0.03 to 38 ng/mL in phosphate buffered saline (PBS), with IC_{50} and LOD values of 0.52 ng/mL and 0.01 ng/mL, respectively. Of all the competitive analogues, the produced mAb exhibited a high cross-reactivity to 17α-nortestosterone (83.6%), the main metabolite of NT in animal tissues. Except for moderate cross-reactivities with trenbolone (22.6%) and β-boldenone (13.8%), the other interference to the assay was negligible (<0.05%). In contrast, the strip test had a visual detection limit of 1 ng/mL in PBS, 2 μg/kg in beef, and 2 μg/kg in pork, respectively, and the results can be judged within 10 min. The ELISA and GC-MS results showed close correlation in beef ($R^2 = 0.9945$) and in pork ($R^2 = 0.9977$). Therefore, the combination of two immunoassays provides a useful screening method for quantitative or qualitative detection of NT residues in animal-origin products.

KEYWORDS: 19-nortestosterone, monoclonal antibody, indirect competitive ELISA, colloidal gold immunoassay, animal tissues

INTRODUCTION

19-Nortestosterone (NT) and its esters are synthetic anabolic steroids which have become widely used as therapeutic agents, mainly in protein deficiency diseases. For example, these drugs have been employed in the therapy of essential asthenia, anemia, osteoporosis, and protein deficiency in the elderly, and in the treatment of mammary carcinoma.[1−3] This anabolic androgenic steroid has also been employed as a doping agent to boost muscular strength and performance in sports and horse racing, and as a growth-promoting agent to accelerate weight gain and improve feeding efficiency in animals.[1,2,4,5] However, NT and its metabolite residues in meat can cause a series of adverse effects, including diabetes mellitus, adrenal atrophy, dyssecretosis, cerebral dysfunction, emotional instability, and even cardiovascular diseases when accumulated in the body at high levels.[6−8]

Many different methods have been described for the determination of NT residues, including gas chromatography coupled to mass spectrometry (GC-MS),[9−12] liquid chromatography coupled to mass spectrometry (LC-MS),[13,14] and other quantitative methods. Chromatographic analyses provide sensitive and specific techniques; however, they usually require highly skilled personnel and expensive equipment. Moreover, these laborious sample pretreatment procedures involve numerous extraction steps that are time-consuming and unsuitable for routine analysis of a large number of samples or on-site determinations. Compared with instrumental methods, immunoassays are portable and cost-effective, with adequate sensitivity, high selectivity, and simple sample extraction processes. Therefore, immunochemical techniques have become popular and are increasingly considered as alternative/or complementary methods for residue analysis nowadays.

Several immunoassays based on the enzyme-linked immunoabsorbent assay (ELISA) for the detection of NT residues in biological matrices have been developed recently.[15−17] An electrochemical immunosensor,[4] a chemiluminescent immunoassay,[5] and a time-resolved fluoroimmunoassay[18] for the analysis of NT have also been established. Compared with ELISA, an immunostrip assay may be an alternative as a rapid screening method because it has several advantages: sample pretreatment can be further simplified; results can be obtained within 5−10 min; and all of the reagents are included in the strip without the need for skilled personnel and instrumentation. Therefore, test stip immunoassays have been developed to detect NT residues recently.[19−21]

However, all of these works have been carried out with polyclonal antibodies (pAbs). Therefore, the objective of this study was (a) to produce a monoclonal antibody (mAb) for the application of NT detection; (b) to test and evaluate this mAb with a one-step strip assay and ELISA for semiquantitative and qualitative NT residue in pork and beef; and (c) validate and compare these results in relation to the confirmatory GC-MS methods.

MATERIALS AND METHODS

Chemicals and Materials. 19-Nortestosterone (NT), 17a-nortestosterone, β-boldenone, estradiol, and trenbolone were purchased from Dr. Company (Germany), while other chemicals were from the China Institute of Veterinary Drug Control (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant
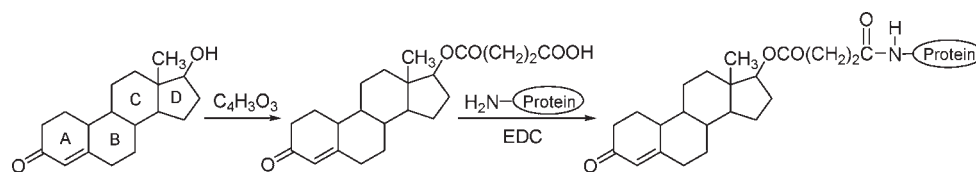
Figure 1. Synthesis procedure for the NT artificial antigen through the EDC method.

(FCA) and Freund's incomplete adjuvant (FIA), hypoxanthine/thymidine/aminopterin (HAT), and hypoxanthine/thymidine (HT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The peroxidase-conjugated goat antimouse IgG (GaMIgG-HRP) was purchased from Sino-American Biotechnology Company (Shanghai, China). RPMI-1640 with L-glutamine was obtained from Gibco. Polyethylene glycol 1500 (PEG 1500) was from Roche Diagnostics Corporation (Indianapolis, USA). Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). Microtiter plates, microculture plates, and cell culture bottles were obtained from Costar Group, Inc. (Bethesda, MD, USA). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, II, USA). Protein G affinity columns were from Amersham Biosciences (Sweden). Nitrocellulose membrane, glass fiber membrane, and absorbance pad were obtained from Millipore (Bedford, MA, USA).

Apparatus. A spectrophotometric microtiter reader (MULTISKAN MK3, Thermo company, USA), provided with a 450 nm filter, was used for absorbance measurements. UV−visible spectra were obtained by using a DU800 Ultraviolet−visible Spectrophotometer (Beckman-Coulter Company, USA). An XYZ Biostrip Dispenser, CM 4000 Cutter, and TSR 3000 membrane strip reader, which were used to prepare test strips, were purchased from Bio-Dot Inc. (Irvine, CA, USA).

Preparation of the Artificial Antigen. The optimized 1-ethyl-3-(3-dimethylaminopropy) carbodiimide (EDC) method[22] was followed to synthesize the immunogen of NT-17-BSA, and the synthetic pathways are presented in Figure 1. Briefly, to a solution of 10 mL of anhydrous pyridine containing 0.36 mmol of NT, 1.8 mmol of succinic anhydride was added and stirred in a dark chamber, and kept at 50 °C for 24 h. Stramineous grease material was acquired after pyridine was removed with a nitrogen evaporator, which was dissolved with 5% NaHCO₃, washed with ether twice, and then acidified by 2 M H₂SO₄. After centrifugation (3000 r/min, 10 min), the supernatant liquid was discarded, and the remains were dried with anhydrous sodium sulfate and recrystallized with ether (20 mL). The amber solid remains are hemisuccinate derivatives of NT.

NT hemisuccinate (0.1 mmol) was suspended in 2 mL of *N,N*-dimethylformamide (DMF) and then 0.1 mmol of *N*-hydroxysuccinimide (NHS) and 0.2 mmol of EDC were added. During the following 24 h, incubation in a dark chamber, the mixture was stirred with a HY-4 reciprocal shaker at 37 °C. After centrifugation, the supernatant was added dropwise to 0.001 mmol of BSA dissolved in 10 mL of PBS (0.01 M, pH 7.4), kept at room temperature. The resulting mixture was stirred by a rotor in a dark chamber at 37 °C for 1 h and then incubated with a reciprocal shaker for 3 h. After centrifugation (3000 r/min, 10 min), the obtained immunogen was dialyzed against distilled water (3 times, 8 h/time) and followed by PBS for 4 days (8 h/time). The coating antigen of NT-17-OVA was prepared by a similar method. An UV absorbance method was employed to determine whether the linking had been a success.

Production of Monoclonal Antibodies. *Immunization Schedule.* Five female BALB/c mice (8−10 weeks old) were injected subcutaneously at multiple points with an NT-17-BSA immunogen (60 μg in 0.1 mL of PBS, mixed with an equal volume of FCA to form an emulsion). For subsequent boosters, FIA was substituted for the FCA as an emulsifier every 3 weeks. After the third booster immunization, the mouse showing the highest anti-NT activity was selected for the fusion

experiments. Four days prior to splenocyte harvest, the mouse was injected with 100 μg of immunogen in PBS, divided equally in intravenous and intraperitoneal injections.

Preparation of Hybridomas. Portions of the cell fusion procedures were described previously by Chen et al.[23] with some modifications. Briefly, the splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing agent, and then the fused cells were distributed in 96-well culture plates supplemented with HAT medium containing 15% FBS with peritoneal macrophages as feeder cells from young BALB/c mice. Ten to fourteen days after fusion, supernatants of hybridoma colonies were screened using a combination of noncompetitive and competitive indirect ELISA for the presence of significant NT recognition activity. Hybridomas secreting highly specific and sensitive anti-NT antibodies were selected and cloned with the method of the limiting dilution for more than three times to get the hybridoma lines. Stable antibody-secreting clones were expanded and stored in liquid nitrogen.

Production and Characterization of Monoclonal Antibodies. Ascite fluids were produced in paraffin-primed BALB/c mice. Purification of mAb was achieved by saturated ammonium sulfate precipitation followed by affinity chromatography on a protein G column. The class and subclass of the isotypes of the purified antibody were determined by using a mouse monoclonal antibody isotyping kit. Measurement of monoclonal antibody affinity (Ka) was carried out according to the procedure described by Wang et al.[24]

Indirect Competitive ELISA Procedures. Bidimensional titration assays were used to determine the most appropriate antibody concentration and the suitable coating antigen concentration for the indirect competitive ELISA (icELISA), and the operation procedure was carried out following the standard schedule.[25] Each well of the microtiter plates was coated with 100 μL of NT-17-OVA (2 μg/mL, in carbonate buffer, pH 9.6) and incubated for 2 h at 37 °C. The free coating antigen was removed with the washing buffer [phosphate buffer (PBS), 0.01 M, containing 0.05% Tween 20], and unbound active sites were blocked with 250 μL blocking buffer [1% (w/v) BSA in PBS] at 37 °C for 1 h. Standards of NT (50 μL each) were added and incubated with 50 μL of anti-NT mAb (diluted 1/10, 000 in PBS) for 25 min at 37 °C. After removing the unbound standards and antibody, 100 μL of GaMIgG-HRP (diluted 1/1000 in PBS) was added and incubated at 37 °C for 25 min. After washing three times, 100 μL of TMB substrate solution was added to each well and incubated for 20 min at 37 °C. The enzymatic reaction was stopped by adding 100 μL of 2 M sulfuric acid per well, and competition curves were obtained by plotting absorbance against the logarithm of analyte concentrations.

Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of NT that produced 50% inhibition of antibody binding to the hapten conjugate. The limit of detection (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition.[26] The dynamic range for the icELISA was calculated as the concentration providing a 20−80% inhibition rate (IC_{20}−IC_{80} values) of the maximum signal. Specificity was defined as the ability of analogues to bind to the mAb, and cross-reactivity (CR) was calculated as (IC_{50} of NT)/(IC_{50} of steroids) × 100.

Colloidal Gold-Labeled Strip Immunoassay. *Preparation of Colloidal Gold.* Colloidal gold with an average diameter of 15 nm was
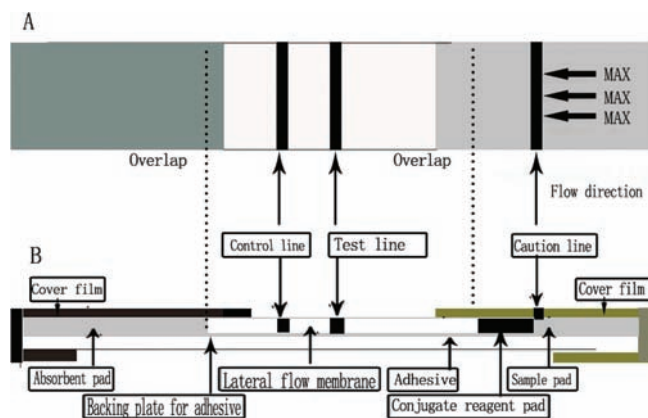
Figure 2. Schematic diagram of a mAb-based NT test strip. (A) Top view; (B) cross-section.

produced by controlled reduction of gold chloride according to the procedure described by Zhao et al.[27] Briefly, 100 mL of 0.01% gold chloride trihydrate solution in super purified water was heated to boiling, and then 2 mL of 1% sodium citrate solution was added while stirring. Gradually, the color changed from light yellow to brilliant red. After the color changed, the solution was boiled for another 5 min to complete the reduction of the gold chloride. The resultant solution was left to cool and recovered to the initial volume with deionized water, and then scanned with UV−vis spectrometry to determine the diameter of colloidal gold particles. The obtained colloidal gold suspensions could be stored at 4 °C for several months, with 0.05% of added sodium azide.

Preparation of Colloidal Gold Labeled mAb. The colloidal gold labeled anti-NT mAb was prepared according to the procedures described before,[27,28] with some modifications. Briefly, the colloidal gold solution was adjusted to pH 9.0 with 0.1 M K_2CO_3. The optimum protein concentration for labeling was determined by the following steps: 25 μL of mAb solution was diluted with 2 mM borate-buffered saline (BBS) (pH 9.0) into gradient concentration, and then 25 μL of colloidal gold solution was added. Mixtures were incubated for 15 min at room temperature, and then 100 μL of 10% NaCl solution was added. The color of samples gradually changed from brilliant red to blue as the concentration of mAb decreased. The optimum concentration of mAb for colloidal gold labeling was the lowest concentration that did not change color, with a 20% increment.

Two milliliters of mAb solution, at the optimum concentration of 2.2 μg/mL, was incubated with 10 mL of colloidal gold solution (pH 9.0) for 30 min at room temperature. After adding 1 mL of 10% BSA solution in BBS, the mixture was incubated at room temperature for another 10 min. BSA helps to further stabilize gold against aggregation and also blocks nonspecific binding sites. The labeled mAb was washed by repeated centrifugation (10,000 r/min) at 4 °C for 30 min with 20 mM BBS containing 1% BSA and 0.1% sodium azide. The precipitate was resuspended in the washing buffer and stored at 4 °C for use.

Assembly of the Testing Strip. A schematic description of the colloidal gold-based one-step strip is illustrated in Figure 2. The strip consisted of three pads (sample, conjugate and absorbent pads) and one nitrocellulose (NC) membrane with test and control zones. NT-17-OVA diluted to 1 mg/mL with carbonate buffer (0.05 M, pH 9.6) and goat antimouse IgG diluted to 0.5 mg/mL with PBS (0.01 M, pH 7.4) were applied in the form of 50 dots/μL/cm to form the test and control lines, respectively. After drying at 37 °C for 60 min, the membrane was blocked with PBS containing 1% BSA and then dried at 37 °C for another 60 min. The treated nitrocellulose membrane was pasted on the center of the plastic backing plate. The conjugate pad was dispensed with

the gold-labeled NT mAb diluted with PBS containing 5.0% (w/v) sucrose, 5.0% (w/v) BSA, 0.8% (w/v) NaCl, and 0.05% (w/v) sodium azide, and then pasted on the plate by overlapping 2 mm with the NC membrane. Sample and absorbent pads were made from nonwoven, 100% pure cellulose fiber. The sample pad was saturated with PBS containing 2.0% (w/v) sucrose, 2.0% (w/v) BSA, 0.5%(w/v) sodium borate, and 0.1% (w/v) sodium azide, and pasted on the same end by its margin justified to the conjugate pad. The absorbent pad was pasted on the other end of the NC membrane by the same overlapping of 2 mm. The whole assembled plate was covered with a color film at both ends and cut into 4 mm width strips. Strips were then sealed in a vacuum-packaged plastic bag containing silica as the moisture absorbent and stored under dry conditions at room temperature for use.

Assay Procedure and Principle. Three drops (about 150 μL) of standard solution or sample extract were added onto the sample pad, and the solution migrated toward the absorbent pad. When NT was absent in the sample, the gold-labeled mAb would be trapped by NT-17-OVA to form a visible test line. When NT was present in the sample, it would compete with the immobilized capture reagent for the limited amount of detection reagent. The more NT present in the sample, the weaker the test line color. If sufficient NT is present, it will completely block the reaction; thus, there is no visible test line on the nitrocellulose membrane. When the test procedure was properly carried out, the control line was always visible, and the whole process would take 5−10 min.

GC-MS Analysis. The GC-MS procedures were followed according to Jiang et al.[29] Briefly, the GC-MS analyses were performed on a DSQ II mass spectrometer (Thermo company, USA) linked to a TRACE GC ULTRA gas chromatograph (Thermo company, USA), equipped with a TR-5MS (Thermo-Fishier, USA) fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas, and the injections were made in splitless mode (1.0 μL) using a TRIPLUS AS autosampler. The ionization mode was positive ion chemical ionization (PCI), and the ion source temperature was maintained at 260 °C. Selected ion monitoring (SIM) with four diagnostic ions (m/z 666, 453, 318, and 306) was used for the screening of individual steroids tested in this study, and full scan mode was followed for confirmation.

Sample Pretreatment Protocols. Fresh meat samples of pork and beef that had not been exposed to anabolic steroids were homo-genized with a high speed triturator, and 2 g of each sample homogenate was accurately weighed into a glass centrifuge tube, then NT standard solution was added. The spiked sample was mixed thoroughly, allowed to stand at room temperature for 2 h, and then a volume of 2 mL acetate buffer (pH 6.5, 0.2 mM) was added. The mixture was subjected to enzymatic hydrolysis with 50 μL of β-glucoronidase from *E. coli* (Sigma-Aldrich, USA) and incubated on an oscillator at 37 °C for 3 h.

For ELISA Analysis. After 2 mL of methanol was added, the sample was mixed briefly and centrifuged (5000 r/min, 10 min). Then 2 mL of this supernatant was diluted with distilled water (1, 2, 5, 10, 20-fold dilution), of which a 50 μL aliquot per well was pipetted into the microtiter plate for analysis.

For Strip Immunoassay. The spiked homogenate was mixed with 2 mL of methanol/water (25:75, v/v), then centrifuged (5000 r/min, 10 min). This suspension was pipetted onto the sample pad for the strip test.

For GC-MS Detection. The homogenate was mixed with 10 mL of methanol and heated at 60 °C for 15 min, then placed in an ice-bath at −18 °C for 2 h. After liquid−liquid extraction with 30 mL of *n*-hexane, the analytes were subjected to a normal-phase SPE C_{18} cartridge for cleanup. The dried organic extracts were derivatized with 100 μL of heptafluorobu-tyric anhydride (Alfa Aesar, USA), and then subjected to GC-MS analysis.

■ RESULTS AND DISCUSSION

Immunogen Synthesis and Conjugation Determination. UV absorbance was used to check the extent of coupling and to
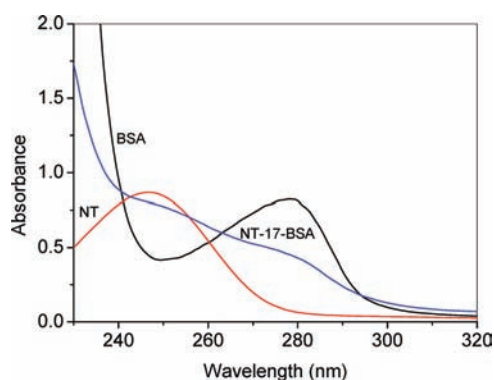
9765

dx.doi.org/10.1021/jf2012437 |*J. Agric. Food Chem.* 2011, 59, 9763–9769

Figure 3. UV—vis spectrum for the artificial antigen of NT-17-BSA.

Figure 4. Optimized standard icELISA inhibition curve for NT. Data were obtained by averaging three independent curves, each run in triplicate. NT-17-OVA (1.0 μg/mL) as coating antigen was prepared in CBS (pH 9.6), purified ascite liquid produced by N1C2E8 hybridoma was diluted 1:10,000 in PBS (pH 7.4), NT was prepared in PBS containing 30% of methanol, and GaMIgG-HRP was diluted 1:1000 in incubation buffer.

determine whether the linking had been a success. The spectra were measured for BSA (1 mg/mL), hapten—protein conjugate (1 mg/mL), and NT (0.1 mg/mL). As seen in Figure 3, an absorbance of NT at 247 nm had shifted from 0.8671 to 0.799 of NT-17-BSA in the UV spectra. Referring to the procedure by Wang et al.,[30] an average hapten/protein conjugation ratio was confirmed as 18.6 for NT-17-BSA. The results were satisfactory values considering that the highest immunogenicity would be produced when the conjugation ratio is between 8 and 25.[31] The corresponding coating antigen of NT-17-OVA gave a similar pattern in UV spectra, and the conjugation ratio was 8.2, which was also satisfactory considering that OVA contains a total of 13 lysine residues.

Monoclonal Antibody (mAbs) Production and Characterization. After three subsequent injections, four mice produced antisera with significant anti-NT activities. From the obtained inhibition curves, the N0.2 mouse afforded the most sensitive IC_{50} value (6.4 ng/mL), that was selected for fusion. The fusion rate was about 86%, and the positive well rate was 21%. Selection of clones from these positive cultures by limiting dilution led to six stable hybridoma cell lines, in which the most sensitive hybridoma named N1C2E8 showed the IC_{50} value of 0.54 ng/mL. This means that the sensitivity of mAb increased approximately 12-fold in comparison to that of the antiserum tested above. Titers of hybridoma supernatant and ascitic fluid determined by indirect ELISA were 1:1000 and 1:80000, respectively. Using a mouse monoclonal antibody isotyping kit, N1C2E8 was determined to be an IgG_1 isotype with a kappa light chain. The affinity constant (K_{aff}) of the selected hybridoma was 3.6×10^{10} L/mol.

Development and Optimization of the icELISA Standard Curve. To ascertain the applicability of the N1C2E8 monoclonal antibody, the icELISA format was investigated. Checkerboard titrations were performed, and the optimal reagent concentrations were determined when the maximum absorbance (A_{max}) was ranging from 1.5 to 2.0, and the dose—response curve of inhibition ratio versus the NT concentration pursued the lowest IC_{50} values. Therefore, optimal concentrations of the coated NT-17-OVA and anti-NT mAb were 1 μg/mL, and 0.5 μg/mL in 1:10,000 dilutions, respectively.

The blocking step is important to avoid nonspecific absorption in the ELISAs; otherwise, unoccupied sites of the plates may absorb the components such as antibody and GaMIgG-HRP during the subsequent steps, which may cause high background. As a result, the blocking buffer containing BSA (1%, w/v) in PBS showed a better result because of the lower background value
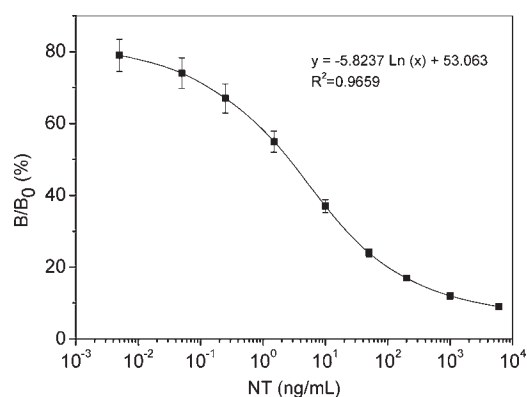
Table 1. Cross-Reactivity (CR) Values of Structurally Related Compounds As Competitors to NT

analogues	IC_{50} (ng/mL)	CR (%)
nortestosterone	0.52	100
17a-nortestosterone	0.62	83.6
trenbolone	2.33	22.3
β-boldenone	3.77	13.8
estradiol	>1040	<0.05
clostebol	>1040	<0.05
testosterone	>1040	<0.05
dihydrotestosterone	>1040	<0.05
epitestosterone	>1040	<0.05
clenbuterol	>5200	<0.01
diethylstilbestrol	>5200	<0.01

(0.13) than that of 1% OVA (0.22) or 2% milk powder (0.18); therefore, it was selected in the following experiments.

The organic solvent added to the assay solution to dissolve the analytes was also evaluated. Increasing the concentrations of methanol generally caused a continuous decrease in the A_{max} value and continuous increase in the time for color development. On the basis of these results and the fact that methanol is commonly used to extract NT residue from food matrices, the optimum combination of methanol/PBS (30:70, v/v) was selected, where A_{max}/IC_{50} was the highest.

To improve the detection process, a rapid one-step competitive assay was substituted for the traditional one described above. The general procedure was followed except for the competitive reaction, for which 50 μL per well of standard solution in PBS and 100 μL per well of a 1:1 (v/v) mixture of mAb and HRP conjugate were added, and the reaction was allowed to occur for 25 min at 37 °C. The IC_{50} and LOD values of the two assay formats were comparable (data not shown); however, the one-step method was more rapid. The optimized icELISA could save 30 min by omitting one washing procedure and shortening the antigen/antibody incubation time, and thus, the analysis time was reduced to about 1.5 h. With this tremendous savings in time,
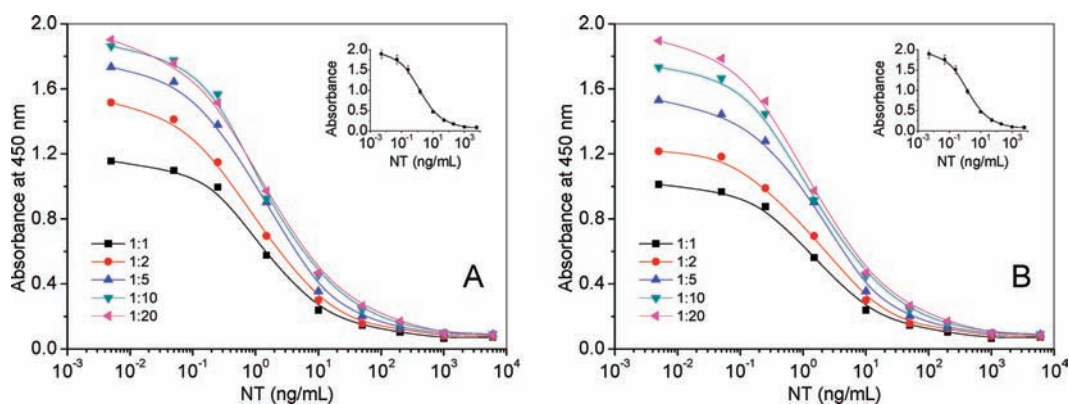
Figure 5. NT inhibition curves in the diluted muscle samples. (A) In beef; (B) in pork. Each point represents the average of three separate assays in triplicate. Insets indicate the icELISA standard curve in PBS.
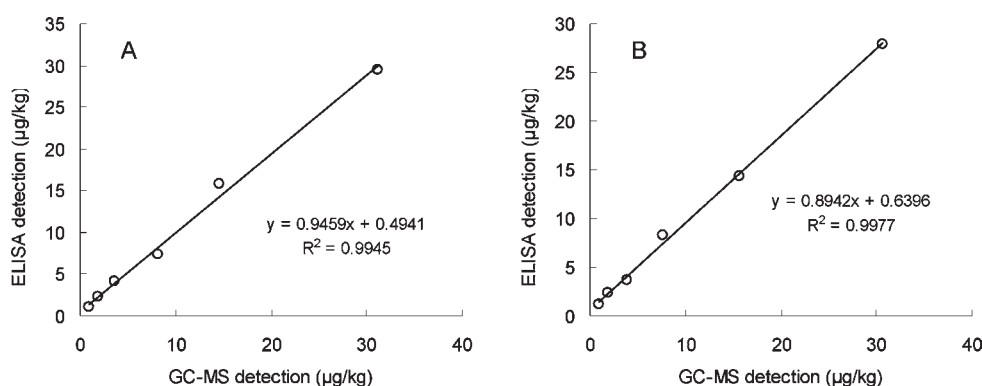

Figure 6. Correlation studies between ELISA and GC-MS detection in muscle samples ($n = 6$). (A) In beef; (B) in pork.

the rapid ELISA provided higher analysis efficiency, and therefore, a representative standard inhibition curve is shown in Figure 4. As can be seen, this assay allowed the detection of NT (20−80% inhibition of color development) from 0.03 to 38 ng/mL, with a LOD value of 0.01 ng/mL. The sensitivity (IC_{50}) of the assay was determined to be 0.52 ng/mL.

Specificity of the Developed Assay. Table 1 shows all the candidate compounds examined in this article. Among the compounds tested, the main cross-reacting chemical was the 17α-epimer of NT, which is known to be a very abundant metabolite in illegally treated animals. Such a high cross-reactivity (83.6%) is not surprising, considering that the NT hapten was coupled to BSA through a hemisuccinate bridge at the C-17 position. It seems that the produced antibody will react with steroids containing a comparable structure, especially those with A and B rings, and changes in the D ring only have a minor influence on the reactivity. Moderate cross-reactivities with trenbolone (22.6%) and β-boldenone (13.8%) were observed in this study. An extra double bond or alkyl group located in cyclopentanoperhydro-phenanthrene is the major difference between those two chemicals and NT, indicating the inhibition binding to the antibody. In the case of other steroids tested (Table 1), little cross-reactivity (<0.05%) was observed. This means that the additional benzene ring, hydroxyl group, and extra chlorine atom in the steroids do make significant changes in the affinity. Similarly, it is not surprising that negligible cross-reactivity with clenbuterol and diethylstilbestrol was observed since the volumes, shapes, and also the electrostatic potentials

of these compounds are dramatically different from those of NT.

Matrix Effects on ELISA. A comparison between calibration plots for NT prepared in PBS and those prepared in animal muscles gave clear evidence of matrix effects (Figure 5). As the dilution of beef extracts increased from 1:2 to 1:20, the absorbance gradually increased to approach the PBS buffer values. It is clear that extracts with low dilution factors (≤5-fold) significantly affected the ELISA curves. The average B_0 values for dilutions at 1:2, 1:10, and 1:20 had absorbencies of 1.515, 1.868, and 1.911, respectively, as compared to 1.896 in PBS. To minimize the effects of physiochemical factors on the ELISA, the final beef extracts need to be diluted at least 10-fold with PBS prior to the assay. When compared to the inhibition curves in pork, similar analytical sensitivity was observed between 1:20 dilution and PBS, indicating that matrix interference was sufficiently low and that 1:20 dilution allowed a significant gain in the detectability of the analyte.

Analytical Characteristics of Test Strip Immunoassay. The results were determined visually by the degree of intensity of the gold color of the test line and the control line on which the NT-17-OVA conjugate and goat antimouse IgG were separately immobilized. The more NT present in the sample, the weaker the test line appears. The sensitivity with the naked eye was defined here as the amount of NT that caused a slight but distinguishable difference compared with the coloration of the negative control. According to the data (not shown), the lowest concentrations of NT were estimated at 1 ng/mL in PBS, 2 ng/mL in beef, and 2 ng/mL in pork, where the color of the test line is

significanly weaker than that of the control line, indicating a positive result. Considering the extraction procedure, the lowest limit of detection in the test strip corresponded to 2 μg/kg in beef and 2 μg/kg in pork. Although the lowest detection limit of the mAb-based immunogold test strip is slightly higher than that of the icELSIA processed with the same antibody, the values are far below 200 ng/mL of the previous studies.[20,21]

Validation of Sample Analysis. Animal samples with different spiked concentrations were analyzed by the test strip and icELISA method, while GC-MS analysis was performed in parallel with the ELISA method for confirmation. For immunoassay strip detection, the results were inconsistent with that of the spiked sample (figure not shown). For ELISA, the spiked concentrations of 1, 2, 4, 8, 16, and 32 μg/kg corresponded to 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ng/mL in beef muscle extracts, and 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 ng/mL in pork muscle extracts, respectively. The resulting correlation coefficients (R^2) between ELISA and GC-MS analysis are shown in Figure 6. It can be seen that an acceptable agreement between the two methods were obtained ($R^2 = 0.9945$ in beef and 0.9977 in pork, respectively); and the results also indicated that the ELISA is reliable for NT residue detection in animal muscles.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +86(373)3040079. Fax: +86(373)3040718. E-mail: jjq5678@126.com.

■ REFERENCES

(1) Bricout, V.; Wright, F. Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances? *Eur J. Appl. Physiol.* **2004**, *92* (1), 1–12.

(2) Sauer, M. J.; Samuels, T. P.; Howells, L. G.; Seymour, M. A.; Nedderman, A.; Houghton, E.; Bellworthy, S. J.; Anderson, S.; Coldham, N. G. Residues and metabolism of 19-nortestosterone laurate in steers. *Analyst* **1998**, *123* (12), 2653–2660.

(3) Massé, R.; Laliberté, C.; Tremblay, L.; Dugal, R. Gas chromatographic/mass spectrometric analysis of 19-nortestosterone urinary metabolites in man. *Biomed. Mass Spectrom.* **1985**, *12* (3), 115–121.

(4) Conneely, G.; Aherne, M.; Lu, H.; Guilbault, G. G. Electrochemical immunosensors for the detection of 19-nortestosterone and methyltestosterone in bovine urine. *Sens. Actuators, B* **2007**, *121* (1), 103–112.

(5) Roda, A.; Manetta, A. C.; Portanti, O.; Mirasoli, M.; Guardigli, M.; Pasini, P.; Lelli, R. A rapid and sensitive 384-well microtitre format chemiluminescent enzyme immunoassay for 19-nortestosterone. *Luminescence* **2003**, *18* (2), 72–78.

(6) Hall, C. E.; Hungerford, S. Similarities and differences between effects of testosterone and 19-nortestosterone in rats, with particular reference to hyper tensogenic potency. *J. Steroid Biochem.* **1982**, *16* (4), 581–585.

(7) Brännvall, K.; Bogdanovic, N.; Korhonen, L.; Lindholm, D. 19-Nortestosterone influences neural stem cell proliferation and neurogenesis in the rat brain. *Eur. J. Neurosci.* **2005**, *21* (4), 871–878.

(8) Bahrke, M. S.; Yesalis, C. E. Abuse of anabolic androgenic steroids and related substances in sport and exercise. *Curr. Opin. Pharmacol.* **2004**, *4* (6), 614–620.

(9) Yamada, M.; Aramaki, S.; Kurosawa, M.; Kijima-Suda, I.; Saito, K.; Nakazawa, H. Simultaneous doping analysis of main urinary metabolites of anabolic steroids in horse by ion-trap gas chromatography-tandem mass spectrometry. *Anal. Sci.* **2008**, *24* (9), 1199–1204.

(10) Bagnati, R.; Fanelli, R. Determination of 19-nortestosterone, testosterone and trenbolone by gas chromatography-negative-ion mass spectrometry after formation of the pentafluorobenzyl carboxymethoxime-trimethylsilyl derivatives. *J. Chromatogr.* **1991**, *547* (1–2), 325–334.

(11) Peterson, H. W.; Christensen, F.; Vad, M. Method validation in relation to accuracy and precision for the gas chromatography-mass spectrometric analysis of 19-nortestosterone in urine with heptafluorobutyric anhydride and N-methyl-N-(trimethylsilyl) trifluoroacetamide. *Analyst* **1994**, *119* (12), 2627–2630.

(12) Ventura., R.; Roig, M.; Pérez, B.; López, S.; Medina, M.; Bosch, J.; Segura, J. Detection of the administration of 17beta-nortestosterone in boars by gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2008**, *22* (12), 1863–1870.

(13) Kim, J. Y.; Choi, M. H.; Kim, S. J.; Chung, B. C. Measurement of 19-nortestosterone and its esters in equine plasma by high-performance liquid chromatography with tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2000**, *14* (19), 1835–1840.

(14) Grace, P. B.; Drake, E. C.; Teale, P.; Houghton, E. Quantification of 19-nortestosterone sulphate and boldenone sulphate in urine from male horses using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2008**, *22* (19), 2999–3007.

(15) Lu, H. H.; Conneely, G.; Crowe, M. A.; Aherne, M.; Pravda, M.; Guilbault, G. G. Screening for testosterone, methyltestosterone, 19-nortestosterone residues and their metabolites in bovine urine with enzyme-linked immunosorbent assay (ELISA). *Anal. Chim. Acta* **2006**, *570* (1–2), 116–123.

(16) Xu, C. L.; Pen, C. F.; Hao, K.; Jin, Z. Y.; Chu, X. G. Determination of 19-nortestosterone residue in aquaculture tissues by enzyme-linked immunosorbent assay and comparison with liquid chromatography and tandem mass spectormetry. *Intern. J. Environ. Anal. Chem.* **2006**, *86* (11), 819–832.

(17) Zhang, Y.; Pan, T.; Fang, G.; Ma, D.; Wang, S. Development of a solid-phase extraction-enzyme-linked immunosorbent assay for the determination of 17beta-19-nortestosterone levels in antifatigue functional foods. *J. Food Sci.* **2009**, *74* (8), T67–74.

(18) Li, X.; Huo, T.; Chu, X.; Xu, C. Time-resolved fluoroimmunoassay for 19-nortestosterone residues in aquaculture tissues. *Anal. Sci.* **2007**, *23* (3), 321–325.

(19) Ploum, M. E.; Haasnoot, W.; Paulussen, R. J.; van Bruchem, G. D.; Hamers, A. R.; Schilt, R.; Huf, F. A. Test strip enzyme immunoassays and the fast screening of nortestosterone and clenbuterol residues in urine samples at the parts per billion level. *J. Chromatogr.* **1991**, *564* (2), 413–427.

(20) Liu, L.; Peng, C.; Jin, Z.; Xu, C. Development and evaluation of a rapid lateral flow immunochromatographic strip assay for screening 19-nortestosterone. *Biomed. Chromatogr.* **2007**, *21* (8), 861–866.

(21) Tian, Z.; Liu, L. Q.; Peng, C. F.; Chen, Z. X.; Xu, C. L. A new development of measurement of 19-nortestosterone by combining immunochromatographic stip assay and ImageJ software. *Food Agric. Immunol.* **2009**, *20* (1), 1–10.

(22) Jiang, J. Q.; Zhang, H. T.; Li, G. L.; Wang, Z. L.; Wang, J. H. Preparation of artificial antigen and immunological trait of 19-nortestosterone. *J. Agric. Biotechnol.* **2010**, *18* (5), 1013–1018.

(23) Chen, Y.; Wang, Z.; Wang, Z.; Tang, S.; Zhu, Y.; Xiao, X. Rapid enzyme-linked immunosorbent assay and colloidal gold immunoassay for Kanamycin and Tobramycin in swine tissues. *J. Agric. Food Chem.* **2008**, *56* (9), 2944–2952.

(24) Wang, Y. C.; Guo, Z. Q.; Li, Y. Z.; Chang, W. B. Production and characterization of anti-estrone monoclonal antibody. *Biomed. Environ. Sci.* **2002**, *15* (2), 103–112.

(25) Chen, L.; Wang, Z.; Ferreri, M.; Su, J.; Han, B. Cephalexin residue detection in milk and beef by ELISA and colloidal gold based one-step strip assay. *J. Agric. Food Chem.* **2009**, *57* (11), 4674–4679.

(26) Wang, L.; Zhang, Y.; Gao, X.; Duan, Z.; Wang, S. Determination of chloramphenicol residues in milk by enzyme-linked immunosorbent assay: improvement by biotin-streptavidin-amplified system. *J. Agric. Food Chem.* **2010**, *58* (6), 3265–3270.

(27) Zhao, Y.; Zhang, G.; Liu, Q.; Teng, M.; Yang, J.; Wang, J. Development of a lateral flow colloidal gold immunoassay strip for the rapid detection of enrofloxacin residues. *J. Agric. Food Chem.* **2008**, *56* (24), 12138–12142.

(28) Zhang, G. P.; Wang, X. N.; Yang, J. F.; Yang, Y. Y.; Xing, G. X.; Li, Q. M.; Zhao, D.; Chai, S. J.; Guo, J. Q. Development of an immunochromatographic lateral flow test strip for detection of β-adrenergic agonist clenbuterol residues. *J. Immunol. Methods* **2006**, *312* (1−2), 27–33.

(29) Jiang, J. Q.; Zhang, L.; Li, G. L.; Zhang, H. T.; Yang, X. F.; Liu, J. W.; Li, R. F.; Wang, Z. L.; Wang, J. H. Analysis of 19-nortestosterone residue in animal tissues by ion-trap gas chromatography-tandem mass spectrometry. *J. Zhejiang Univ., Sci., B* **2011**, *12* (6), 460–467.

(30) Wang, S.; Allan, R. D.; Skerritt, J. H.; Kennedy, I. R. Development of a class-specific competitive ELISA for the benzoylphenylurea insecticides. *J. Agric. Food Chem.* **1998**, *46* (8), 3330–3338.

(31) Singh, K. V.; Kaur, J.; Varshney, G. C.; Raje, M.; Suri, C. R. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjugate Chem.* **2004**, *15* (1), 168–173.