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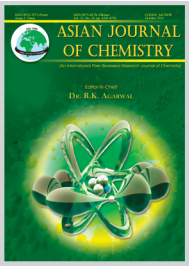

ELISA and LC-MS/MS Method for Detecting 19-Nortestosterone Residue in Animal Tissues

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Two different analytical methods for the detection of 19-nortestosterone (NT) residue in bovine have been developed and the comparison was also performed. For this purpose, EDC method was employed to synthesize the artificial antigen of NT-17-BSA. The results of IR and UV-visible spectra indicated that the artificial antigen was synthesized successfully and the conjugation ratio was 18:1. Based on the checkerboard titration results, an icELISA standard curve was established. The linear range was from 0.04 to 86 ng/mL, with LOD and IC_{50} value of 0.02 ng/mL and 1.2 ng/mL, respectively. For LC-MS/MS analysis, analytes were separated using a mobile phase solution of 1 % formic acid in water/acetonitrile/methanol (60:20:20, v/v/v) at a flow rate of 0.2 mL/min. The ultraviolet detector was operated at 242 nm and the injection volume was 10 µL. Positive chemical ionization mode was used and the relative collision energy was optimized to 28 %. When applied in spiked bovine samples, the correlation coefficients (R_2) of the icELISA and LC-MS/MS data were 0.9963 in muscle, 0.9988 in liver and 0.9984 in kidney, respectively. The results suggest that it was an advantage through coupling of icELISA as screening method and LC-MS/MS as confirmatory method for detecting 19-nortestosterone residues in animal edible tissues.

Key Words: 19-Nortestosterone, Artificial antigen, Indirect competitive ELISA, Liquid chromatography tandem mass spectrometry, Bovine, Detection.

INTRODUCTION

19-Nortestosterone (17β-hydroxy-19-norandrost-4-en-3-one, NT), is one kind of synthetic anabolic steroids (ASs) known as far back as the 1930s and also endogenous chemicals found in a number of species recently[1]. 19-Nortestosterone is used in veterinary as well as in human medicine for the treatment of protein deficiency diseases, osteoporosis and advanced breast cancer[2-4]. This anabolic androgenic steroid has also been employed as a doping agent to boost muscular strength and performance in sport and horse racing and as a growth-promoting agent to accelerate weight gain and improve feeding efficiency in animals[5,6]. Studies show 19-nortestosterone and its metabolites residues in meat from husbandry animals produce a series of adverse effects, including jaundice, tumor to liver organ, cerebral dysfunction and emotional instability[7,8].

So the use of natural and synthetic hormones for growth promotion purpose in meat-producing animals has been prohibited in the European community since 1986[9]. 19-Nortestosterone and its esters, as well as metabolites are also prohibited in the food animal veterinary drugs and other banned compounds list in china (notice no. 193 of the ministry of agriculture in April 2002). However, illegal use of 19-nortestosterone as a growth promoter has been widely reported throughout Europe[4] and in china, thus prompting continuous surveillance to control its abuse.

Traditionally, 19-nortestosterone residue analysis has relied upon classical analytical methods, such as gas chromatography coupled to mass spectrometry (GC-MS)[10-14], liquid chromatography coupled to mass spectrometry (LC-MS)[15-17] and other quantitative methods. LC-MS/MS provides a universal detector, since anabolic steroids may be analyzed without derivatization. Recently, LC-MS/MS has been successfully applied to the analysis of anabolic steroids in various biological samples including urine from bovine and horse, bovine hair and fat[18-21], but no applications of 19-nortestosterone residue detection on muscular tissue of bovine have been reported.

Compared with chromatographic methods, immunoassays are portability and cost-effective, with adequate sensitivity, high selectivity and simple sample extraction process. Therefore, immunochemical techniques become a popular and are increasingly considered as alternative/ or complementary methods for residue analysis nowadays. The objective of the present study is to develop an indirect competitive ELISA method for the determination of 19-nortestosterone residues in bovine tissues (muscle, liver and kidney) and to compare

it with liquid chromatography tandem mass spectrometry technique.

EXPERIMENTAL

19-Nortestosterone was purchased from Dr. Company (Germany); β-glucuronidase from *E. coli* was obtained from Sigma-Aldrich (USA); SPE C_{18} columns were purchased from Dalian Sipore Co., Ltd (China); BSA, OVA and GaRIgG-HRP were purchased from Sino-American Biotechnology Company (Shanghai, China); FCA, FIA and EDC were obtained from Pierce; NHS was from Japan, MSDS available; O-(carboxymethyl) hydroxylamine hemihydrochloride and succinic anhydride were supplied by Sigma while dialysis bag was from Solarbio Company; TMB, phenacetin and urea peroxide were obtained from Sigma Company. All other solvents, reagents and chemicals were of analytical grade or HPLC grade, unless otherwise stated.

A spectrophotometric microtitre 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); Infrared spectra were acquired with an IR spectrometer (TENSOR 27, Bruker Company, Germany); The LC-MS/MS analysis were performed on a surveyor liquid chromatograph (Thermo Company, USA), equipped with a PDA Plus Detector (Thermo-Finnigan, USA) and a Thermo ODS-HYPERSIL column (3 μm, 2.1 × 150 mm); Mass spectrometry was performed using a mass spectrometer equipped with a Turbo Ion Spray ESI source (LCQ Deca XP MAX, Finnigan Company, USA).

Synthesis of artificial antigen for 19-nortestosterone: To a solution of 10 mL anhydrous pyridine containing 100 mg (0.36 mmol) of 19-nortestosterone, 180 mg (1.8 mmol) of succinic anhydride was added and stirred in dark chamber, kept at 50 ºC for 24 h. Stramineous grease material was acquired after pyridine removed with nitrogen evaporator, which was dissolved in 5 % $NaHCO_3$, washed with ether twice and then acidified by H_2SO_4. After centrifuged, the supernatant liquid was discarded; the remains were dried with anhydrous sodium sulfate and recrystallized with ether. The amber solid remains are hemisuccinate derivative of 19-nortestosterone.

EDC method was employed to synthesis the artificial antigen of NT-17-BSA and the procedure is presented in Fig. 1. Briefly, 37.5 mg (0.1 mmol) of 19-nortestosterone hemisuccinate was suspended in 2 mL DMF and then 12 mg (0.1 mmol) of NHS and 38 mg (0.2 mmol) of EDC were added. During the following 24 h incubation in dark chamber, the mixture was stirred with a HY-4 Reciprocal Shaker at 37 ºC. After centrifuged, the supernatant was added dropwise to 66 mg (0.001 mmol) of BSA dissolved in 0.01 M PBS (pH 7.4). The resulting mixture was stirred by rotor in dark chamber at 37 ºC for 1 h and then incubated with a reciprocal shaker for 3 h. After centrifuged at 3000 rpm for 10 min, the obtained supernatant was dialyzed against distilled water and followed by PBS for 4 days. When the absorption peak of the dialyzed solution disappeared, checked by UV-Visible spectra, stored the artificial immunogen in ampoule, kept at -20 ºC. A NT-17-OVA coating antigen was prepared in a similar way.

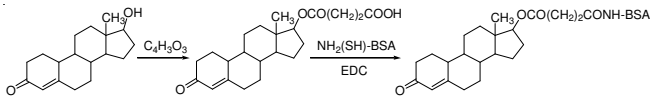

Fig. 1. Synthesis procedure for the artificial antigen of NT-17-BSA through the EDC method

Identification of artificial antigen: For UV-VIS analysis, 0.1 mg/mL of 19-nortestosterone, 1 mg/mL of BSA and NT-17-BSA were prepared with PBS, which UV-visible spectra were recorded and the conjugation rate of artificial antigen was calculated according to Huang *et al.*[22]. The IR identification was performed as follows. Heated under an infrared lamp, 1 mg of NT-17-BSA and 10 mg of KBr were even mixed and grinded in an agate mortar. Taking part of the mixture into a mold, at the pressure of 10 t and keeping it 3-5 min, a transparent KBr pellet with the thickness of 1 mm was made, which infrared spectra were acquired with a IR spectrometer.

Preparation of anti-19-nortestosterone polyclonal antibody: Three female New Zealand white rabbits were subcutaneously immunized at four sites in the back with NT-17-BSA conjugate. FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every 28 days with 500 μg of immunogen and blood samples were taken for ELISA identification from the marginal vein of the ear 10 days after each immunization. Eight days after the final boost, all rabbits were exsanguinated by heart puncture and the antiserum was prepared by allowing the blood to clot overnight at 4 ºC, followed by centrifugation with 10000 rpm for 20 min to remove particulate materials. The crude serum was purified by saturated ammonium sulfate (SAS) precipitation method, then aliquotted and stored at -70 ºC.

Establishment of icELISA standard curve: The indirect ELISA and indirect competitive ELISA (icELISA) procedures were performed as Hao *et al.*[23]. Checkerboard titration method was used to optimize the coating antigen and the primary antibody concentrations. Sensitivity was evaluated according to the inhibition rate and the data were calculated using the IC_{50} values, which represented the concentration of 19-nortestosterone that produced 50 % inhibition of antiserum binding to the hapten conjugate. The detection of limit (LOD) was defined as the lowest concentration that exhibits a signal of 15 % inhibition[24]. 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.

Preparation of samples: In this study, we follow the reported methods[25,26], with significant modification. Briefly, 10 g fresh bovine samples (muscle tissue, liver and kidney) were homogenized with a high speed triturator and collected in a 50 mL round-bottomed plastic flask. For spiking tests, standard solutions of 19-nortestosterone in methanol-water (60:40, v/v) were added at this step. The spiked sample was mixed thoroughly, allowed to stand at room temperature for 2 h and then a volume of 10 mL acetate buffer (pH 5.2, 0.2 mol/L) was added. The mixture was subjected to enzymatic hydrolysis with 50 μL of β-glucoronidase and incubation at 37 ºC for 6 h. After centrifugation at 3000 rpm for 10 min, the supernatant was divided into two parts. One aliquot was

diluted 1:10 with PBS for ELISA detection; the other aliquot was mixed with 10 mL of methanol and heated in a water-bath at 60 ºC for 15 min, then placed in an ice-bath at -18 ºC for 2 h. The mixture was centrifuged at 3500 rpm for 5 min and this procedure was repeated one more time. The supernatant was extracted twice with 10 mL *n*-hexane to remove fat and the aqueous methanol layer was transferred into a calibrated flask and then submitted to solid-phase for clean-up process.

Separation and clean-up of crude extract: The SPE C_{18} cartridges were consecutively conditioned with 5 mL of methanol and then 5 mL of deionized water at a flow rate of 0.3 mL/min. After drying for 5 min under vacuum, the cartridges were loaded with the aqueous extract solution (weak vacuum applied, approx. 700 mbar). The cartridge was then washed with 20 mL of the elution solution [*n*-hexane-ether (70:30, v/v)] at a flow rate of less than 0.5 mL/min. Next, the eluate was washed with 2 mL of 1.0 M sodium hydroxide. After centrifugation (3500 rpm, 5 min), the organic layers were collected in a tapered glass tube and the solvents were removed under a stream of nitrogen in a water bath at 45 ºC. The extracts were redissolved in methanol for further analysis.

LC-MS/MS analysis: Analytes were separated using a mobile phase solution of 1 % formic acid in water/acetonitrile /methanol (60:20:20, pH 2.5) at a constant temperature of 30 ºC and a flow rate of 0.2 mL/min. The ultraviolet detector was operated at 242 nm and the injection volume was 10 µL. Positive chemical ionization (PCI) mode was used and the relative collision energy was optimized to 28 %. A pseudo-molecular ion $[M + H]^+$ was selected as the parent ion for fragmentation and the corresponding fragment ions were used for identification and quantitation.

RESULTS AND DISCUSSION

UV-VIS spectrogram of artificial antigen: UV-VIS spectrum (Fig. 2) showed that the maximum absorbance of 19-nortestosterone and BSA were at 247 nm and 278 nm respectively, while that of the artificial antigen changed significantly. This confirmed that 19-nortestosterone had been conjugated to BSA successfully. Calculated according to Huang *et al.*[22], molar ratio of NT-17-BSA conjugate was obtained.

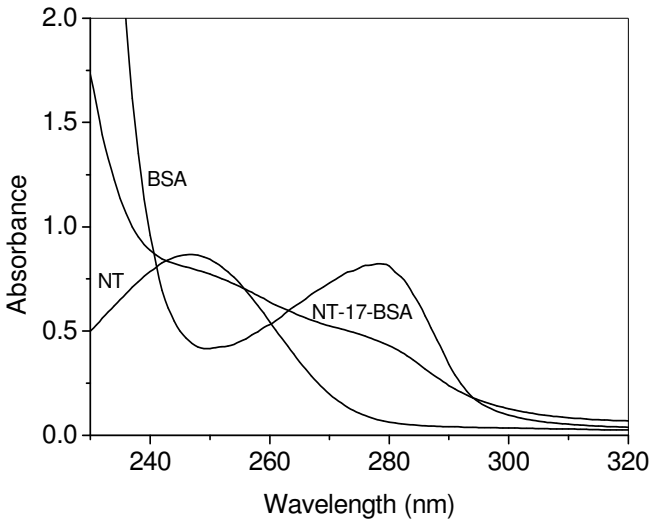

Fig. 2. UV-VIS spectrum for the artificial antigen of NT-17-BSA

IR identification: Fig. 3 shows the IR spectra of artificial antigen. The infrared absorption bands were extremely similar in 3200-2500 cm^{-1} and 1660-1500 cm^{-1} between BSA and NT-17-BSA, which were the characteristic absorption peak of amino acid in BSA, showed the BSA character in the artificial antigen. As compared to NT-17-BSA, a strong sharp absorption peak at 3300 cm^{-1}, which attributed to hydroxyl group, was appeared only in C-17 of 19-nortestosterone, proved the derivatization and conjugation had been successful. The absorption peak at 1700 cm^{-1} and 1460-1380 cm^{-1}, which ascribed to carbonyl and methyl group respectively, were enhanced obviously when 19-nortestosterone conjugated to BSA. This showed the two characteristic functional groups of 19-nortestosterone were remained in the artificial antigen. The results indicated that the artificial antigen was synthesized successfully.

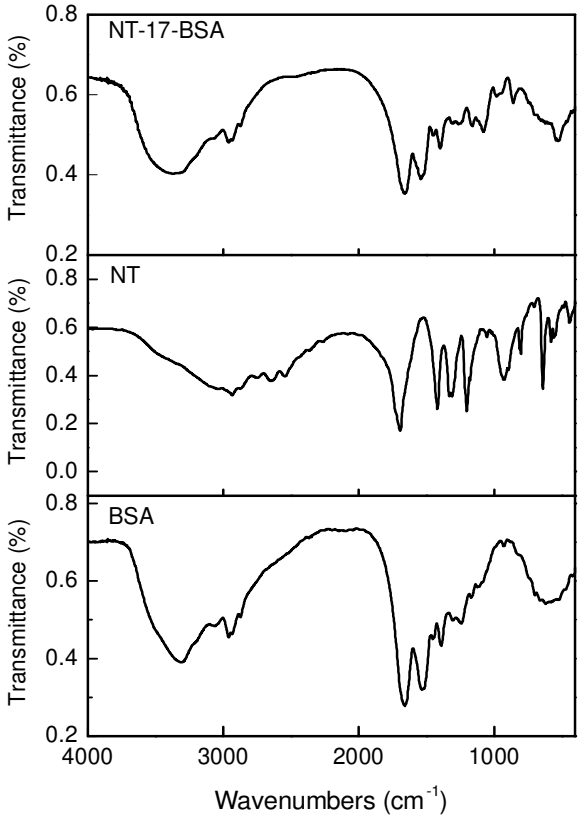

Fig. 3. IR spectra of BSA, NT and NT-17-BSA

Indirect competitive ELISA standard curve: Checkerboard titrations were performed, taking into account the optimal dilutions. The optimal reagent concentrations were determined when the maximum absorbances (A_{max}) were between 1.5 and 2.0 and the dose-response curve of inhibition ratio *versus* the 19-nortestosterone concentration pursued the lowest IC_{50} values. From the checkerboard assays, a representative standard inhibition curve is shown in Fig. 4.

As can be seen, the optimum concentration of coating antigen was 1 µg/mL and pAb was 1:10,000 dilutions. This assay allowed the detection of 19-nortestosterone (20-80 % inhibition of colour development) from 0.04 to 86 ng/mL, with an IC_{50} value of 1.2 ng/mL. The limit of detection (LOD) of the assay was 0.02 ng/mL.

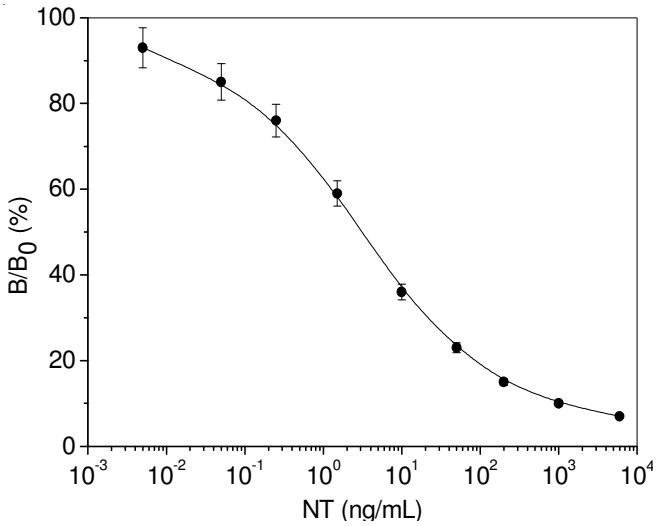

Fig. 4. Optimized standard icELISA inhibition curve for 19-nortestosterone. Data were obtained by averaging three independent curves, each run in triplicate. NT-17-OVA (1 µg/mL) as coating antigen was prepared in CBS (pH 9.6), purified anti-serum produced by NT-17-BSA as immunogen was diluted 1:10 000 in PBS (pH 7.4), 19-nortestosterone was prepared in PBS, containing 10 % methanol; GaRIgG-HRP was diluted 1:1000 in incubation buffer

LC-MS/MS detection: In the LC-MS/MS procedure, quantitative analysis of 19-nortestosterone was performed using the external standard method by comparing the chromatographic peaks of the spiked samples with those of 19-nortestosterone standard. Fig. 5 presents the example of chromatogram obtained from 19-nortestosterone; with retention time (RT) of the target substance was 4.76 min. The mass spectral properties of 19-nortestosterone in positive chemical ionization are displayed in Fig. 6. Based on the mass spectrum data in Fig. 6, four diagnostic ions (m/z 275, 257, 239 and 231) were determined.

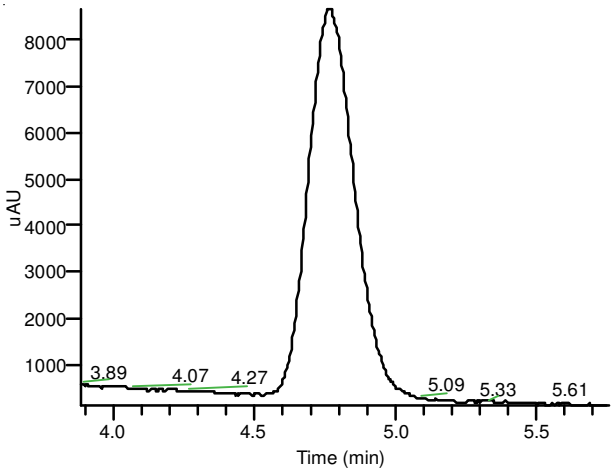

Fig. 5. Ion chromatogram of 19-nortestosterone (RT = 4.76 min) in a positive bovine sample

Correlations between icELISA and LC-MS/MS analysis: The objective of this part was to compare results obtained from ELISA with those from LC-MS/MS to determine the effectiveness of the developed icELISA as a screening tool to reduce the number of samples that need to be analyzed by LC-MS/MS. Fig. 7 displays the performance of the ELISA in

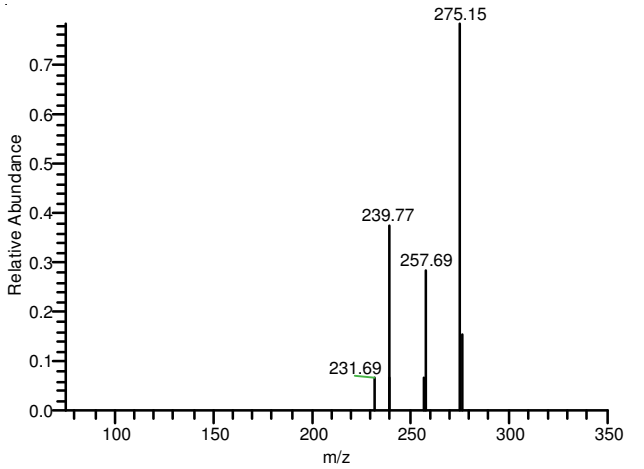

Fig. 6. ESI (+) MS/MS spectrum of 19-nortestosterone. The relative collision energy was 28 %

comparison with the LC-MS/MS method and the linear regression between the two group data. From Fig. 7 we can find that the data spots are nearly distributed on both sides of the standard line, that is to say, the data obtained from these two methods are very similar and with no significant differences. The correlation coefficients (R^2) of the icELISA and LC-MS/MS data were 0.9963 in muscle, 0.9988 in liver and 0.9984 in kidney, respectively.

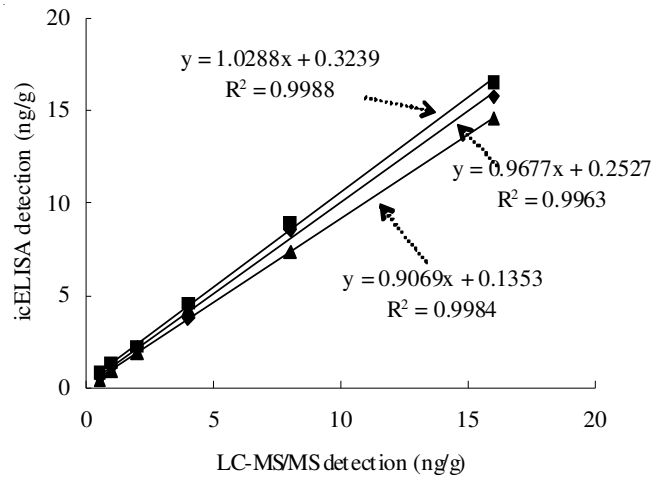

Fig. 7. Comparison between icELISA and LC-MS/MS methods in spiked bovine samples. (◆) muscle (■) liver (▲) kidney

Conclusion

An optimal icELISA and modified LC-MS/MS method to quantify 19-nortestosterone in bovine have been developed and those two methods have proven to be suitable for detecting veterinary drug residues in foodstuffs. The results demonstrate this ELISA can be used as a screening method and provides a noticeable advantage over methods requiring a tedious sample cleanup procedure. Therefore, this assay has the potential to be incorporated into a quantitative monitoring program for the rapid screening of 19-nortestosterone residue in food. To ensure rapid, effective and reliable results in relation to meat control for the residue program in connection with the EC directive (86/469/EEC)[9], any positive samples would then be submitted for further investigations, using the LC-MS/MS

procedures. Since the percentage of negative samples is usually high, the combination of two analytical methods presents a practical advantage over monitoring 19-nortestosterone residues in animal edible tissues.

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