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Development and optimization of an indirect enzyme-linked immunosorbent assay for 19-nortestosterone*

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ABSTRACT

A polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) has been developed, optimized and validated to measure this anabolic steroid. Influence of several physicochemical parameters, such as incubation time, ionic strength, detergent concentration and pH were selected to provide a highest sensitivity on the ELISA format. The regression equation of the final inhibition curve was: $y = -0.3194x + 1.6316$, $R^2 = 0.9927$. The linear range was between 0.1 and 25 ng/ml and the IC_{50} was 3.5 ng/ml. The specificity was evaluated by five structurally related anabolic steroids, and none of them had significant cross-reactivity. Finally, the accuracy and precision of this assay were evaluated by means of spiked samples. The recovery was between 76.9 and 104.7%, and the variation coefficient was between 5.2 and 13.4%.

KEY WORDS: 19-nortestosterone, ELISA, immunoassay

INTRODUCTION

19-Nortestosterone, also called nandrolone (NT), one of the most powerful anabolic steroids, has been widely used in veterinary as well as human medicine for the treatment of protein deficiency diseases, osteoporosis and male contraception (Hobbs et al., 1996; Kuhnz and Gieschen, 1998; Bruggeman et al., 2003; Meriggiola

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et al., 2003). Nandrolone has also been employed as a growth-promoting agent to accelerate weight gain, improve feeding efficiency in meat producing animals and as a doping agent to boost muscular strength and performance in sports and horse racing (Kim et al., 2000; Kohler and Lambert, 2002).

As their possible harmful effects result from the intake of hormone residues and their metabolites, usage of growth-promoting drugs for fattening livestock have been banned in many countries. However, illegal use of 19-nortestosterone as a growth promoter has been widely reported in many countries (Draisci et al., 2000; Le Bizec et al., 2000). Thus, it is necessary to control 19-nortestosterone's abuse.

Conventional chromatographic methods, such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS) and tandem mass spectrometry (MS-MS), are used as confirmatory methods because of the high specificity of the information from the analyte (Le Bizec, 1999; Reznik et al., 2001; Robinson et al., 2001; Marcos et al., 2004). But those methods are time-consuming and expensive when large number of samples is detected. Thus, radioimmunoassay and enzyme immunoassays, which based on immunoassay, have been widely used for routine screening for their cost-effective and field-portable (Degand et al., 1989; Cooper et al., 2001).

Some authors (Evrard et al., 1986; Van Look et al., 1991; Roda et al., 2003) have reported the preparation steps of the antibodies for the small molecule immunizing hapten (oximation, conjugation, immunization, etc.). However, the development and optimization of the indirect enzyme-linked immunosorbent assay for 19-nortestosterone has not been reported yet. An indirect competitive ELISA assay for the measurement of NT has been developed and its optimization also been reported in this article. The concentration of the immunoreagents and the influence of incubation time, ionic strength, detergent concentration and pH were selected to provide the highest sensitivity on the indirect ELISA format.

MATERIAL AND METHODS

Reagents and chemicals

Bovine serum albumin (BSA, electrophoretic grade) and ovalbumin (OVA, electrophoretic grade) were purchased from Boao Biotechnology Company (Shanghai, China); 19-nortestosterone, testosterone, medroxyprogesterone, complete Freund's adjuvant (FAC), incomplete Freund's adjuvant (FAI) were purchased from Sigma (St.Louis, USA). Epitestosterone, dehydroepiandrosterone and progesterone were kindly gifted by Chinese Academy of Inspection and Quarantine; goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase

(HRP) and Tween-20 were obtained from Shanghai KangChen Bio-tech; isobutyl chloroformate, N, N-dimethylformamide and tri-n-butylamine were obtained from Feixiang Chemical Plant (Shanghai, China); 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Longyou Chemical Company (Zhejiang, China).

Apparatus

Polystyrene microtiter plates were purchased from Nunc (Roskilde, Denmark); MuLtiska Mks microplate reader was purchased from Thermo Labsystem (Helsinki, Finland); the LC/MS spectrometer (Waters Platform ZMD 4000) was obtained from Waters Company (Milford, MA), the shaker from Taicang Science and Education Equipment Company, UV-2100 UV scanner from Ruili Company (Beijing, China) and PHS-3TC pH meter from Shanghai Tianda Apparatus Company.

Buffer and solutions

The assay buffer was 0.01 M phosphate buffer, and if not indicated otherwise, the pH was 7.5. Coating buffer was 0.05 M carbonate-bicarbonate buffer, pH 9.6. The buffer PBST was PBS with 0.05% Tween 20. Citrate buffer was 0.15 M sodium citrate in water at pH 5.5. Antibody dilution buffer was 0.1% gelatin in PBS. The chromogen solution was 3,3′,5,5′-tetramethylbenzidine (TMB 0.42 mg/ml) in glycol and the substrate solution was H_2O_2 (0.006%) in citrate buffer. Enzymatic reaction is stopped by 2 M H_2SO_4.

Standard solutions and sample preparation

Nandrolone (52 mg) standard solutions were dissolved in 100 ml dehydrated alcohol and then diluted with PBS buffer containing 10% methanol to provide a series of standards solutions of NT (0, 0.1, 0.3, 0.9, 2.7, 8.1, 25 ng/ml.)

19-Nortestosterone is illegally used as a growth promoter in pig raising in China. In order to determine the content of 19-nortestosterone in pork, normal and well-muscled cattle presented for slaughter at local meat plants were purchased. Fat and connective tissue in the sample was removed, and then the sample was homogenized. Exactly 2 g of the sample was distributed into 6 ml PBS buffer containing 10% methanol, followed by carbonate-bicarbonate buffer (6 ml) and ethyl acetate (10 ml). The mixtures were shook vigorously for 30 min, and then centrifuged at 4000 rpm for 10 min. The supernatant was pipetted into another centrifugal screw-cap vial and evaporated to dryness under a gentle nitrogen stream. The residue was dissolved in 100 µl of methanol by ultrasonication for 30 s, and 900 µl PBST was added following. The solution was employed in the ELISA assay.

Steroid conjugate production

The 19-nortestosterone-3-carboxymethoxylamine conjugate (19-NT-3-CMO) was prepared as follows. To a solution of 19-nortestosterone (27 mg) in dry pyridine (10 ml), carboxymethoxylamine hemihydrochloride (219 mg) was added. The pyridine was removed by vacuum distillation at 50°C for 30 min. Then the residue was dissolved in ethyl acetate (50 ml). The organic layer was washed four times with distilled water (20 ml), dried with anhydrous sodium sulphate, and then removed by vacuum distillation. After that, the resulting white foam was recrystallized with diethyl ether, and then 19-NT-3-CMO as a white powder (210 mg) was obtained. The scheme of steroid conjugate production was shown on Figure 1. The oximated production was characterized by mass spectrum.

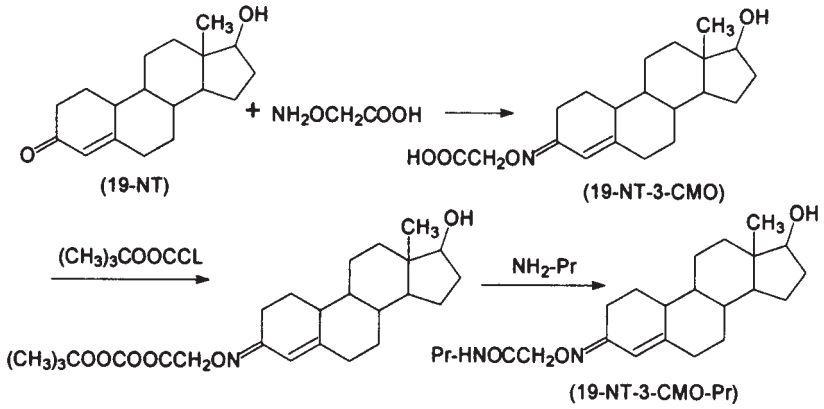

Figure 1. Synthetic pathway used for the preparation of steroid conjugate (19-NT-3-CMO-Pr)

The immunogen (19-NT-3-CMO-BSA) and the coating conjugation (19-NT-3-CMO-OVA) were prepared by mixed-anhydride method. 100 mg of 19-NT-3-CMO was dissolved in 1 ml N, N-dimethylformamide (DMF) and tri-n-butylamine (15 µl) was added in together with isobutyl chloroformate (45 µl) at 4°C for 2 h. To a solution of protein (BSA:218 mg, OVA:120 mg) in 2 ml PBS, DMF (1 ml) was added and stirred at 4°C for 1 h. With gentle stirring, the active hapten was slowly dripped into the protein solutions. The reaction mixture was stirred at 4°C for 4 h, then dialysed against distilled water. The NT conjugates were aliquot into 100 µl volumes and stored at -20°C.

Immunization protocol

New Zealand white rabbits (one-month-old) were immunized subcutaneously various sites with 1 mg of the conjugate in water emulsified with complete Freund's

adjuvant, with booster injections being given at 3 week intervals in incomplete adjuvant. Test blood was withdrawn from the marginal ear vein and monitored for the presence of antibodies, every 7 days after each booster injection. Antiserum R1, R2, R3, R4 and R5 with adequate titer, affinity and specificity was obtained 3 months after the first immunization. The serum was harvested 7 days after the last booster injection, the serum with eqal volume of glycerol was aliquoted, and stored at -20°C. All animal experiments have a permission of Jiangsu Animal Management Committee in China.

ELISA procedure

Microtiter plates were coated with 19-NT-3-CMO-OVA (from 1/1000 to 1/32,000, in coating buffer, 100 µl/well) for 2 h at 37°C. Then, the plates were washed three times with PBST (200 µl/well, 3 min), and blocked with OVA (2% in PBS, 200 µl/well) for 2 h at 37°C. Plates were washed as described above and 100 µl aliquots of the mixture of NT 0 standard and the diluted serum (from 1/1000 to 1:128,000, in antibody dilution buffer) was added and incubated for 30 min at 37°C. The plates were washed again, and a solution of goat anti-rabbit IgG-conjugated to HRP (anti IgG-HRP, 1/3000 in antibody dilution buffer), was added (100 µl/well) and incubated for 30 min at 37°C. After washing, 100 µl mixture of the chromogen and the substrate (1:10, v/v) was added and incubated for 15 to 30 min at 37°C before the enzymatic reaction was stopped by adding 2 M H_2SO_4 (100 µl/well). The absorbance was read at 450 nm by a MuLtiska Mks microplate reader.

Optimization of the ELISA method

A set of experimental parameters (incubation time, ionic strength and pH, detergent concentration, etc.) was studied sequentially to improve the immunoassay sensitivity, and study the immunoassay performance under several conditions (Oubina et al., 1999).

These experiments were carried out using the indirect protocol described above. In this case, seven NT standards (from 0 to 25 ng/ml) added duplicates were used to prepare calibration curves. The coating antigen NT-OVA diluted to 1/8000 in coating buffer and the antiserum diluted to 1/8000 in antibody dilution buffer were the immunoreactive chosen as already described. On the same plate, each curve varied on one of the investigated parameters. The best experimental conditions on each step were chosen to evaluate the next parameter in the following order.

The NT standard solutions and the diluted serum were aliquot added and incubated in each well. On each microtiter plate, the mixtures were incubated for different periods (10, 20, 30, 40, 50, 60, 90 min) at 37°C. Finally, the plates were washed and processed as already described.

Different concentrations of PBS (0.05, 0.02, 0.01, 0.005 M) were prepared and used to dilute the antibody. The different competitive experiments were studied in the same microtiter plate.

PBS buffers ranging from pH 2.5 to pH 10.5 were used to prepare solutions of the immunoassay and to carry out the competitive immunoassay. PBS buffer containing different concentrations of Tween 20 (0.2, 0.1, 0.05, 0.005, 0.001, 0.0005, %) were used to wash the plates in the competitive ELISA.

The diluted serum and NT standard solutions were added and incubated at different volume ratio (70/30, 60/40, 50/50, 40/60, 30/70, v/v, 100 µl/well). On each microtiter plate, the mixtures were incubated for 30 min at 37°C.

The antibody dilution buffer containing different concentrations of goat anti-rabbit IgG, conjugated to HRP (1/1000, 1/2000, 1/3000, 1/5000, 1/800, v/v), were added (100 µl/well) and incubated for 30 min at 37°C.

Different volume ratios of the chromogen and the substrate (1/3, 1/5, 1/8, 1/10, 1/12, v/v) were prepared and used in the chromogenic reaction step (100 µl/well) at 37°C.

The chromogen and the substrate were mixed as volume ratio described already. The mixtures incubated during different periods (5, 10, 15, 30, 60 min) at 37°C. Finally, the enzymatic reaction was stopped by adding 2 M H_2SO_4 (100 µl/well) and the absorbance was measured at 450 nm with MuLtiska Mks microplate reader.

Optimized competitive ELISA

The final analytical procedure was as follows: microtiter plates were coated with 19-NT-3-CMO-OVA (1/8000, in coating buffer, 100 µl/well) for 2 h at 37°C and then the plates were washed with PBST (3 times, 200 µl/well). The wells were blocked with OVA (2% in PBS) for 2 h at 37°C. Then the plates were washed again, and the NT standards (0.1 to 25 ng, in PBST) or samples were added to the coated plates (50 µl/well), followed by the diluted antiserum (1/8000 in antibody dilution buffer, 50 µl/well). After incubation (30 min, 37°C) and washing steps, a solution of anti-IgG-HRP (1/3000 in antibody dilution buffer) was added (100 µl/well) and incubated for 30 min at 37°C. After washing, the mixtures of chromogen and substrate were added (1:5, v/v, 100 µl/well) in the plates. The enzyme reaction was stopped after 15 min at 37°C with 2 M H_2SO_4 (100 µl/well) and the absorbance was measured at 450 nm.

Cross-reactivity

Stock solutions of five steroids (testosterone, epitestosterone, progesterone, dehydroepiandrosterone and medroxyprogesterone) were prepared in methanol. Standard curves for each of these compounds were constructed (1.76 nM to

36 μM, in PBST) and their IC_{50} values were determined by the optimized ELISA. The cross-reactivity values were calculated according to the following equation:

$$\text{Cross-reaction rate (\%)} = IC_{50}(\text{NT}) \times 100\%/IC_{50}(\text{aim chemical})$$

Precision and accuracy

The precision study was performed using bovine tissue samples spiked with NT at different concentrations (1 or 5 ng/g). Analyses of these samples were performed in duplicates in separate plates over five different days. The values of the coefficients of variation were measured. Briefly, accuracy was determined by adding known amounts of standard NT dose (1 and 5 ng/g) to bovine tissue samples as already described and all samples were assayed by the optimized ELISA and the recovery values calculated.

RESULTS AND DISCUSSION

The LC-MS of 19-nortestosterone and 19-nortestosterone-3-carboxymethoxylamine conjugate (19-NT-3-CMO) were shown in Figure 2. Two prominent fragment ions seemed to be generic to 19-nortestosterone, namely m/z 274 and 272. The major fragment ions at m/z 348 and 346 were thought to be a result from MH^+ and MH^- for the 19-NT-3-CMO.

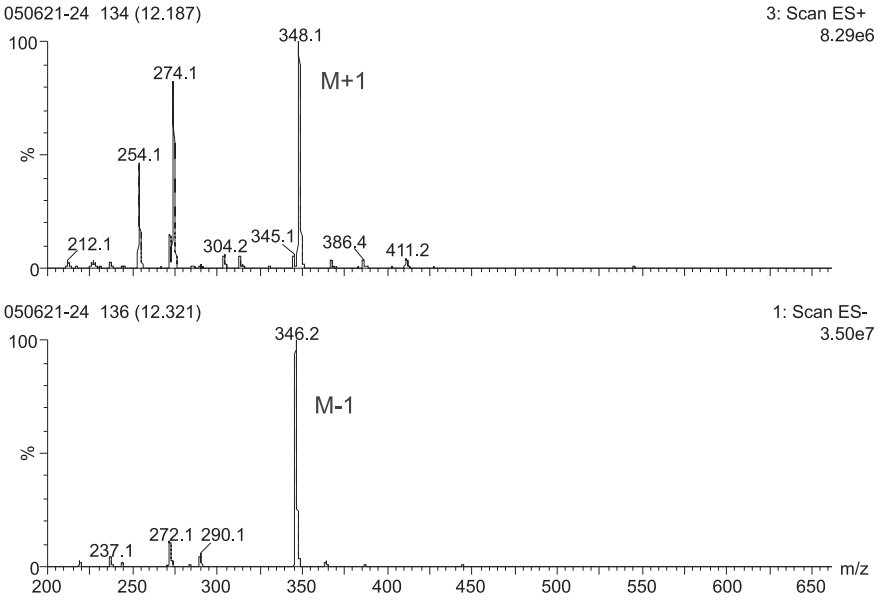

Figure 2. Determination results of the NT and its oximated production by MS

Antiserum of the 19-NT-3-CMO-BSA was evaluated by indirect competitive ELISA. Antiserum titration, by definition, corresponds to the antiserum dilution resulting in uninhibited assay signal three times the background signal under given assay conditions. The titrations for R1, R2, R3, R4 and R5 were 1:51,200, 1:25,600, 1:102,400, 1:204,800 and 1:51,200, respectively, which indicated that the coupling of 19-NT-3-CMO-BSA was successful. As R4 displayed the highest titer, our subsequent experiments were carried out with the R4 antiserum.

The coated concentrations of 19-NT-3-CMO-OVA (from 1/1000 to 1/32,000, in coating buffer) and the diluted serums (from 1/1000 to 1:128,000, in antibody dilution buffer) were evaluated by indirect non-competitive ELISA. The concentration corresponds to the dilution resulting in the A_{max} at 1.5 under given assay conditions. For this reason, the optimum concentrations of the coating conjugate and the antiserum were both 1/8000.

It has been reported (Oubina et al., 1999), the time given to the immunoreagents to interact, may have a direct effect on the sensitivity of the immunoassay. For this reason, the effect of the incubation time was studied. And the immunoassay features were readily influenced by the duration of the competitive step (10, 20, 30, 40, 50 and 60 min). Figure 3 showed the variation of the IC_{50} and the maximal absorbance

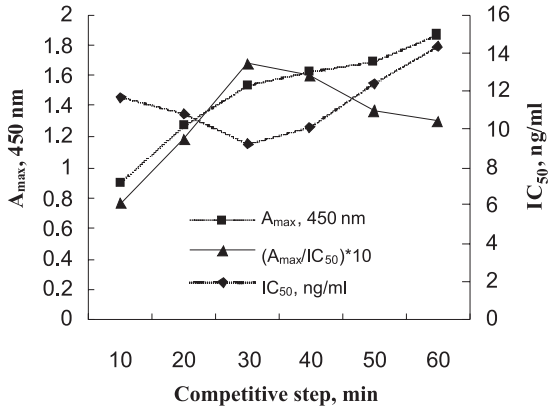

Figure 3. Influence of the length of the competitive step on IC_{50} and A_{max} of the NT immunoassay

under the investigated conditions. An increase of the IC_{50} value was observed when varying the incubation time from 30 min to 1 h. The maximum absorbance value of the assay also varied from 0.9 to 1.8 units when increasing the incubation time. As it can be observed in the Figure 3, 30 min of incubation was sufficient for adequate sensitivity of the assay without diminishing the signal (A_{max}/IC_{50} ratio).

This parameter had a strong effect on the NT immunoassay (see Figure 4). Significant changes were observed in both the IC_{50} value and the maximal absorbance of the assay. The best sensitivity was recorded at the PBS concentration of 0.01 M.

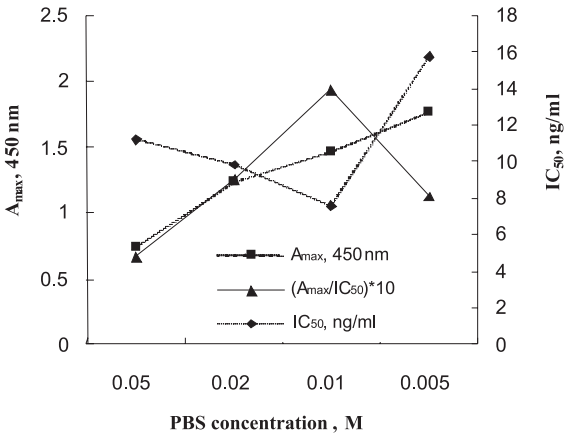

Figure 4. Influence of the length of the ionic strength on IC_{50} and A_{max} of the NT immunoassay

Concentration below this value (0.005 M of PBS), showed a clear increase of the IC_{50} and. In contrast, concentrations above 0.01 M (0.02 and 0.05 M of PBS) inhibited the assay by drastically reducing the absorbance. The salts would thus compete with the antibody, establishing electrostatic interactions with the analyte and consequently diminishing the detectability of the assay.

To evaluate this effect, antibody dilution buffer were prepared at pH values varying from 2.5 to 10.5. As it is shown in Figure 5, the best A_{max}/IC_{50} ratio was obtained at pH around 7.5. Below pH 7.5, the maximum absorbance decreased and at pH 2.5 reached a value as low as around 0.05 units. At pH values higher than 8.5, the absorbance also decreased, however, although the maximal absorbance at pH 10.5 was of 0.7 units only, it was still possible to observe a competitive assay with good sensitivity. The pH value may affect the ionization, not only of the

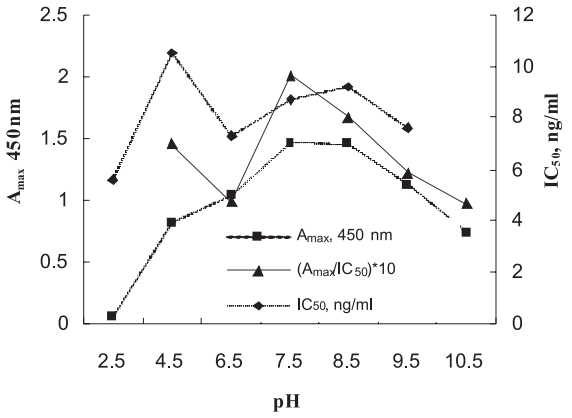

Figure 5. Influence of the length of the pH on IC_{50} and A_{max} of the NT immunoassay

analyte, but also of other immunoreactive species participating in the competitive ELISA. As a result, noncovalent interactions such as hydrogen bonding or electrostatic interactions, that stabilize the immunocomplexes will be affected and subsequently influence the immunoassay sensitivity.

Tween 20 is a non-ionic detergent commonly used in immunoassay techniques to reduce non-specific bindings. Figure 6 showed the variation of the IC_{50} and maximal

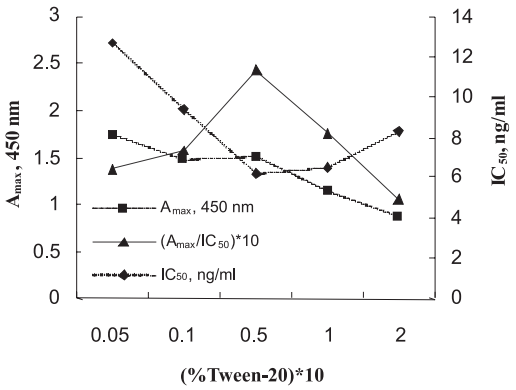

Figure 6. Influence of the detergent Tween 20 on IC_{50} and A_{max} of the NT immunoassay

absorbance of the immunoassay when varying the concentration of this component of the buffer. The best A_{max}/IC_{50} ratio was obtained when using a buffer containing 0.05% Tween 20. Below this concentration the absorbance varied slightly, but when exceeding this concentration the absorbance of the assay decreased drastically.

To evaluate this effect, the diluted serum and NT standard solutions were added and incubated in each well at different volume ratio (70/30, 60/40, 50/50, 40/60, 30/70, v/v, 100 µl/well). As it was shown in Figure 7, a clear decrease of the IC_{50} and of the maximum absorbance was observed when varving the volume ratio

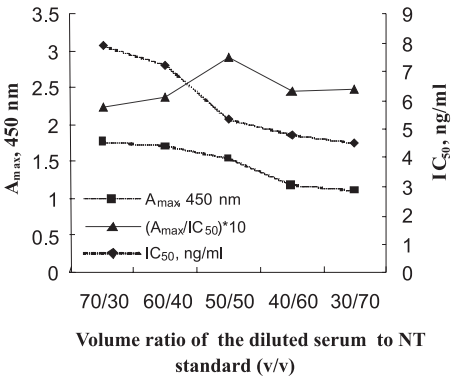

Figure 7. Influence of the volume ratio of the diluted serum to NT standard (v/v) on IC_{50} and A_{max} of the NT immunoassay

from 70/30 to 30/70. The best Amax/IC_{50} ratio was obtained at the volume ratio around 50/50, which was chosen to establish the final immunoassay protocol.

The goat anti-rabbit IgG, conjugated to HRP was diluted with antibody dilution buffer at different concentrations (1/1000, 1/2000, 1/3000, 1/5000, 1/8000, v/v). Figure 8 showed the variation of the IC_{50} and maximum absorbance of the immunoassay when varying the concentration of this antibody. The maximum absorb-

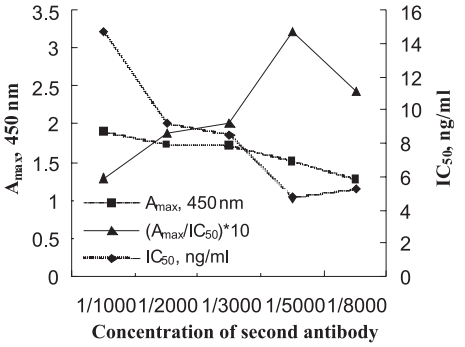

Figure 8. Influence of the concentration of second antibody on IC_{50} and A_{max} of the NT immunoassay

ance has a clear decrease when reducing the concentration from 1/1000 to 1/8000, while the values of IC_{50} were decrease by the ratio, too. The best A_{max}/IC_{50} ratio was obtained at the concentration 1/5000 and was chosen to establish the final immunoassay protocol.

To evaluate this effect, the mixtures of chromogen and substrate were added and incubated in each well at different volume ratio (1/3, 1/5, 1/8, 1/10, 1/12, v/v, 100 µl/well). As it was shown in Figure 9, the IC_{50} showed a clear increase when varying the volume ratio from 1/3 to 1/12 but the absorbance was reduced,

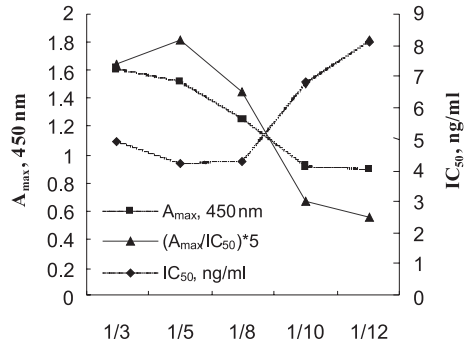

Figure 9. Influence of the volume ratio of chromogen to substrate (v/v) on IC_{50} and A_{max} of the NT immunoassay

simultaneously. The best A_{max}/IC_{50} ratio was obtained at the volume ratio around 1/5, which was chosen to establish the final immunoassay protocol.

The effect of competitive time was evaluated at different reaction time (5, 10, 15, 30 and 60 min). The immunoassay features were readily influenced by the chromogenic reaction step. Figure 10 showed the variation of the IC_{50} and the maximal absorbance under the investigated conditions. The best sensitivities were obtained with short incubation periods for this step. An increase of the IC_{50} value was observed when prolonged the length of this step. As it can be observed in the Figure 10, 15 min of incubation was sufficient for an adequate sensitivity of the assay without diminishing the signal.

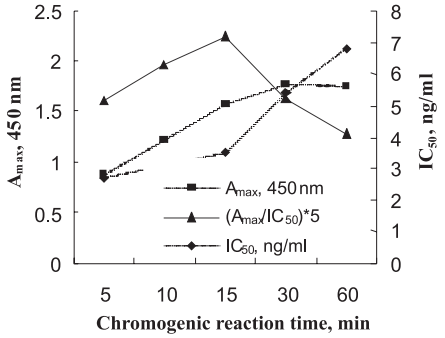

Figure 10. Influence of the length of the chromogenic reaction step on IC_{50} and A_{max} of the NT immunoassay

Under optimum physicochemical conditions, a typical competitive displacement binding curve of NT was shown in Figure 11. The binding curves analysed by immunoassay reported here had an IC_{50} of 3.5 ng/ml, and the working range was placed between 0.1 and 25 ng/ml. The standard curve was:

$$y = -0.3194x + 1.6316, R^2 = 0.9927$$

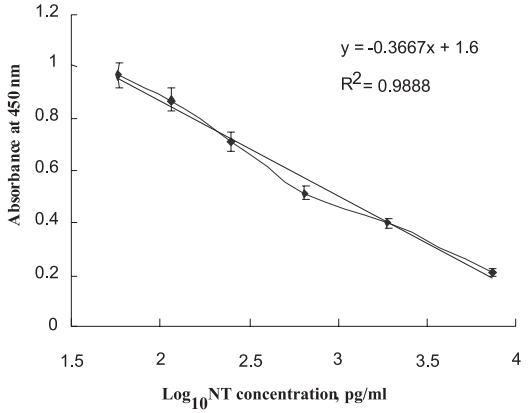

Figure 11. Calibration curve obtained for NT using optimized ELISA

The maximum signal of the assay was always around 1.5 units of absorbance. The sensitivity reached by this immunoassay proved sufficient for analysing samples and superior to the previously reported immunoassay for NT (Cooper et al., 1998).

The optimized immunoassay was evaluated to determine its selectivity by five structurally related steroids (testosterone, medroxyprogesterone, epitestosterone, progesterone and dehydroepiandrosterone). The results of the cross-reactivity studies were shown in Table 1. It can be observed that in spite of the important chemical similarities between the immunogen and steroids, the specificity of this assay was really high. From the five compounds evaluated, only two were slightly recognized. The rest of the compounds showed insignificant cross-reactivity demonstrating a high specificity of this assay.

Table 1. Cross reactivity of NT antiserum with its structurally related steroids

Compounds	Cross-reaction, %
19-Nortestosterone	100
Testosterone	1.3
Medroxyprogesterone	0.1
Androstenedione	<0.1
Epitestosterone	<0.1
Dehydroepiandrosterone	<0.1
16, 17-Dihydronortestosterone	<0.1
Norepitestosterone	<0.1
Progesterone	<0.1

The intra-assay precision of the analytical method was evaluated by analysing five times inside a plate, and the samples spiked with NT at different concentration levels.

Similarly, these samples were analysed on different days using the same protocol to obtain the precision between different assays. Table 2 showed the results obtained from these experiments. It can be observed that the intra-assay and

Table 2. Intra-assay and inter-assay precision and accuracy for NT determination in spiked samples

Spiked amount ng/ml	Intra-assay, n=5			Inter-assay, n=5		
	mean ± SD	CV, %	recovery, %	mean ± SD	CV, %	recovery, %
1	1.05 ± 0.055	8.5	104.68	0.96 ± 0.08	13.4	96.4
5	4.06 ± 0.126	5.2	79.3	3.83 ± 0.215	9.3	76.9

the inter-assay coefficients of variation are 5.2 and 9.3% when measuring around the middle point of the standard curve. The values were a bit higher if measurements take place at the limits of the working range of the assay, but always the co-

efficients of variation obtained were below 15%. The accuracy was evaluated by adding known amounts of standard NT dose (1 and 5 ng/g) to bovine tissue samples and compared the measured value by the optimized ELISA with the recovery values calculated. Table 2 showed the recoveries obtained at different concentration levels. It can be observed that the accuracy ranged from 76.9 to 104.68% at different NT concentrations spiked samples. The recovery values were a bit low if measurements take place at the high concentrations spiked.

CONCLUSIONS

A fast, easy to perform, sensitive and specific polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the measurement of nortestosterone has been developed and optimization. The strategy used in this paper is to develop an immunoassay that improving the immunoassay sensitivity. The assay enables to process a large number of samples within a short period of time and does not require highly skilled personnel. This immunoassay can complement chromatography techniques in field assay conditions and screening procedures, nevertheless, more studies should be performed for its application in commercialization.

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