Determination of Anabolic Steroid Residues (Medroxyprogesterone Acetate) in Pork by ELISA and Comparison with Liquid Chromatography Tandem Mass Spectrometry

PENG CHIFANG, XU CHUANLAI, JIN ZHENGYU, CHU XIAOGANG, AND WANG LIYING

ABSTRACT: Medroxyprogesterone acetate (MPA) has a relative molecular mass of only 344.5 and it has no immunogenicity. The analytical methods for the carbodiimides and the mixed anhydride were both adopted to couple MPA with bovine serum albumin, the carrier protein. The coupling rates of conjugate using the above methods were estimated to be 14 and 20 by ultraviolet spectrophotometry. The coupling was successful and verified according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. New Zealand White rabbits were immunized with the conjugate, the coupling rate of which was 14, and blood was collected after 5 periods of immunities. Then the titer of antiserum was tested to be 2.6 × 105 by indirect enzyme-linked immunosorbent assay (ELISA). Based on the purified antibody, a competitive indirect ELISA was developed. ELISAs provided a limit of detection of 0.096 ng/g, recoveries (in the edible tissues) between 72% and 91%, and a working range of 0.1 to 8.1 ng/g. Preliminary evaluation of assay performance through specificity, sensitivity, precision, and accuracy showed that this ELISA method can be applied to the practical detection of MPA in tissue samples. Moreover, it was compared with liquid chromatography tandem mass spectrometry (LC/MS/MS). The ion pair for quantification of MPA was 345.2/123.1, and the linear equation of MPA was $Y = 6.68 \times 10^3 X + 6.63 \times 10^2$. The 2 analytical methods can be applied to monitor MPA and other steroid residues in edible foods.

Keywords: coupling, determination, medroxyprogesterone acetate (MPA), residues, pork tissues, enzyme-linked immunosorbent assay (ELISA), liquid chromatography tandem mass spectrometry

Introduction

Medroxyprogesterone acetate (MPA) is a synthetic hormone that belongs to the gestagen class. It often substitutes as a prophylactic in human medicine and is widely used in the treatment of hormone-dependent tumors. Among veterinary drugs, MPA is considered a hormone that can speed up fattening (Bernard 1971). To test MPA residues by ELISA, the 1st thing is to prepare antibody related to MPA, which must have good specificity and high biological titer. The relative molecular mass of MPA is only 344.5 and it has no immunogenicity; it cannot induce antibody formation. Protein coupling is necessary (Choi and others 2000).

The 2 methods of determination of anabolic steroid residues in animal muscle tissues are liquid chromatography tandem mass spectrometry (LC/MS/MS) and tandem mass spectrometry as well as gas chromatography-mass spectrometry (GC/MS) (Daeseleire and others 1991; Draisci and others 1998; Philippe and others 1999; Choi and others 2000; Stolker and others 2002; Rambaud and others 2005). Sample preparation is trivial (Rapp and Meyer 1989; Yu and others 2005).

The objective of the present study was to develop a method of determining medroxyprogesterone acetate residues in pork tissues by using enzyme-linked immunosorbent assay (ELISA), and to compare it with high-performance liquid chromatography and tandem mass spectrometry. This article describes, to our knowledge, for the 1st time an inexpensive, simple, rapid, and reliable ELISA for pork tissue MPA. The method is potentially applicable for other gestagen or gestagen metabolite determinations.

In the development of ELISA, the key step is the synthesis of the MPA complete antigen (Lewis and others 1992). In our research, MPA was coupled with bovine serum albumin (BSA) in 2 different ways: the carbodiimides (method A) and the mixed anhydride (method B) procedures. Then the coupling rates of the 2 compounds were tested separately. Experiments of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and animal antibodies show that the synthesis of MPA complete antigen was quite successful, which lays a good foundation for the development of a MPA testing kit using ELISA.

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Materials and Methods

Reagents

The following were used: MPA and epitestosterone (ETS) standard sample were purchased from Sigma (St. Louis, Mo., U.S.A.); All the other steroids used were supplied by Riedel-deaen (Seelze, Germany). BSA and ovalbumin (OVA) (Sino-American Biotechnology Co., Shanghai, China, imported in bulk), water-soluble carbodiimide (Sigma); Coomassie Brilliant Blue G250 (Shanghai Boao Biotech Co., Ltd., Shanghai, China); hydroxylamine HCl (Shanghai Chemical Agent Co., AR, Shanghai, China); succinic anhydride (Kunshan Niansha Auxiliary Agent Factory, CP, Shanghai, China); N,N-dimethylformamide (Wuxi Chinese Traditional and Western Medicine Group Co., Ltd., AR, Wuxi, China); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI); 1,4-dioxane; triethylamine (Shanghai Chemical Agent Co.,

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AR); tributylamine (Shanghai Chemical Agent Co., CP); isobutyl chlorocarbonate (Shanghai Feixiang Chemical Factory, CP), methanol, ethyl acetate, isopropanol, perchloric acid, and sodium borate, analytical or HPLC grade (Sigma), and phosphate-buffered saline (PBS) (Shanghai Chemical Reagent Station, AR).

Apparatus

The following were used: Electronic balance AB104-N (Shanghai Mettler Toledo Group, Shanghai, China); Electro-Thermostatic blast oven (Shanghai Yuejin Medical Instruments Factory, Shanghai, China); PHS-3TC acidimeter (Shanghai Tianda Apparatus Co., Ltd., Shanghai, China); auto double-layer quartz distillatory (Ronghua Apparatus Manufacturing Co., Ltd., Jintan, Jiangsu); U-3000 UV scanner (Shimadzu, Tokyo, Japan); 722-style UV-Vis-NIR spectrophotometer (Shanghai Analytical Instrument Overall Factory, Shanghai, China); Bio-Rad mini gelatin cataphoresis apparatus (American Bio-Rad Co., Hercules, Calif.); FTS EI585-Q freeze dryer (American Stone Ridge; New York, N.Y.); Multiscan MK3 Luminometer, transferpipette, tips, and 8 × 12 Microlon ELISA plates were gotten from Thermo Labsystems (Hong Kong, China).

An Agilent 1100 series LC system (Wilmington, Delaware, U.S.A.) including G1313A quaternary pump, G1313A autosampler, and G1316A column oven were used for all analyses. All analytes were separated using a 150 × 2.1-mm SUPELCO Discovery® C_{18} column (Supelco, Bellefonte, Pa., U.S.A.) with 5-μm particle size. A binary gradient consisting of acetonitrile (A) and purified water (B) at a flow rate of 0.3 mL/min was used. Injection volume of 10 μL was used for all analyses (Fluid Management System Inc., Minneapolis, Minn., U.S.A.). The gradient was as follows: for androgens and progestogens the linear gradient protocol was 50% A to 100% A within 15.0 min. Mass spectrometry was performed using an API3000 tandem triple-quadrupole mass spectrometer equipped with a TurboIonSpray source (HP Lab, Palo Alto, Calif., U.S.A.). The ion spray voltage was 3000 V. High-purity nitrogen was used as nebulizer, heater, curtain, and collision gases. Heater gas was set at 7.5 L/min and the TurboIonSpray probe temperature was maintained at 550 °C. The nebulizer and curtain gases were, respectively, 12 and 8 L/min, whereas the gas pressure in the collision cell was set at 3.4×10^5 Torr. MRM was used for the multiple product ions of each analyte. Precursor/daughter ions were set to unit resolution and dwell time was 150 ms.

MPA-BSA conjugate

Method A. At the oximation, MPA (85 mg, about 0.25 mol) was dissolved in 20 cm^3 of anhydrous ethanol and hydroxylammonium chloride (21 mg) was dissolved in 10 cm^3 of double-distilled water. Then the 2 solutions were blended and reacted in an ice bath for 2.5 h during which about 7 cm^3 of NaOH (0.05 mol cm^{-3}) was dripped in gradually. White deposit appeared after acetate-buffer (5 cm^3) and chipped ice (about 25 mg) was added. Then the solution was placed at 4 °C for a day (Wang 1997).

The solution above was centrifuged and its supernatant was discarded. The white deposit at the bottom, after centrifugation, was dissolved in 20 cm^3 of N, N-dimethylformamide succinic anhydride (50 mg). After reaction at room temperature for 2 h, triethylamine (100 μL) was added and then allowed to react again for an hour (Wang 1997).

Coupling reaction of MPA and BSA. BSA (500 mg) and EDCI (100 mg) were dissolved with 10 cm^3 of PBS (0.01 mol dm^{-3}, pH 8.0) and then the above acylate was dripped gradually into the solution. EDCI (50 mg) was added after an hour's reaction. The solution was then agitated in the dark, allowed to stand for a night and dialyzed against double-distilled water for the next 2 d, with 4 times changing the water at intervals. Finally, it was freeze-dried and preserved.

The method of MPA-OVA (ovalbumin) conjugation is the same as the previously described method A of MPA-BSA conjugation.

Method B. The oximation and acylation of MPA are the same for the 1st several steps. After reaction with triethylamine for an h, tributylamine (360 mm^3) and isobutyl chlorocarbonate (280 mm^3) were added, and then again allowed to react for an h at 4 °C.

1, 4-Dioxane (1 cm^3) and BSA (500 mg) were dissolved in 8 cm^3 of distilled water, and then the above acylate was dripped gradually into the solution. The solution was reacted for 4 h at 4 °C. The end product was dialyzed for 48 h at 4 °C, during which the dialysate was changed 4 times, the PBS solution for the 1st 2 times, and then distilled water was added. The next process was free-drying and preservation.

Identification of conjugate

Identification of acylate. The entire reaction system, which changed from clarity to white deposit, shows the production of oximation. TLC was applied after acylation with succinic anhydride.

According to the TLC result, the chromatographic band of acylated MPA had a distinct tailing phenomenon compared with that of original nonacylated MPA, and the band of the former was above. This analysis shows the product of acylation.

Electrophoresis analysis of conjugate. The fact that the reaction system changed from clarity to white deposit when colorless acylated solution was added to the clear BSA solution identifies the production of MPA-BSA conjugate (Zhou and Hu 2003).

Coupling rate of MPA-BSA formation

Spectrophotometry was adopted to determine the coupling rate of MPA-BSA (Wang and Fan 2000).

First the content of the protein in the conjugate was tested by the method of Bradford (Wang 1997) and Lowry (Wang and Fan 2000). Although the conjugate was constituted by 2 kinds of molecular protein-hapten, the absolute content of conjugate was often expressed by the relative content of protein, due to the fact that the 2 contents were consistent. So, here the Bradford method was used to test the protein content.

Then BSA standard solution was prepared with the same concentration as the above resultant concentration. Different concentrations of MPA standard solutions (10 μg cm^{-3}, 20 μg cm^{-3}, 25 μg cm^{-3}, and 30 μg cm^{-3}) were prepared and tested using the UV-3000 scanner. It showed that 247 nm was the maximum wavelength and the absorbance was tested under this wavelength.

The absorbances of BSA and MPA-BSA solutions were tested separately under the maximum wavelength of MPA. Afterward, the coupling rate was calculated according to the Lambert-Beer law and the formulas were referred to as by Choi and others (2000).

A series of MPA standard solutions(10 μg cm^{-3}, 20 μg cm^{-3}, 25 μg cm^{-3}, and 30 μg cm^{-3}) were prepared and scanned by UV-scanner (Yang and others 1998).

Coupling rate of conjugate coupled by the carbodiimides

1. Concentration of protein in the conjugate was tested by the method of Bradford and then the conjugate, according to the result, was diluted to 2 different concentrations: 26 mg cm^{-3} and 65 mg cm^{-3}.

2. BSA standard solutions with the same concentrations as the above solution were prepared separately.

3. The above BSA solutions were tested through UV scanning and the maximum wavelength of BSA was determined to be 278 nm.

4. Absorbances of the 4 solutions mentioned above were tested at 247 nm, the maximum wavelength of MPA. When water was used as the control, the absorbances of ethanol (20%) at l = 247 nm and 278 nm were 0.0944 and 0.0501, respectively, which indicated that the error was acceptable when water was used as solvent. So ethanol (20%) could be replaced by water.

Coupling rate of conjugate coupled by the mixed anhydride. The coupling rate of conjugate coupled by the mixed anhydride was tested by the same way as mentioned in the previous case. But here, after the determination of the concentration of protein in the conjugate, the concentrations of MPA-BSA and BSA were taken as 30 μg cm^{-3} and 60 μg cm^{-3}. They were then scanned by a UV scanner.

Immunization of animals

Three New Zealand female white rabbits were immunized with MPA-BSA conjugate synthesized by the methods of carbodiimides using the intradermal-intravenous route (Bradford 1976; Draisci and others 1998). MPA-BSA lyophilized powder was dissolved with physiological saline solution to make a solution of 1 mg cm^{-3}. The titers of antiserum were determined by ELISA. Antibodies were purified by ammonium sulfate precipitation followed by further purification using a Protein-A-Agarose affinity chromatography column. Antibody activity in the fractions were verified by ELISA. Pool fractions that showed maximum activity were stored at –80 °C in small portions (0.5 cm^{-3}) (Shen and Zhou 1998).

The determination procedures of the titer of antiserum by ELISA were carried out as follows.

1. Microtiter plates (96 wells) were coated with the solution of MPA-OVA (Ovalbumin) (0.2 g dm^{-3}), 100 mm^3 each well, and placed in the refrigerator for over night at 4 °C.

2. Microtiter plates were then blocked with the solution of OVA (20 g dm^{-3}), 200 mm^{-3} each well, and kept for 2 h at 37 °C.

3. Four microtiter strips were prepared. The dilution of antibody was added in the 1st row as control, the s row was for negative serum (f_r = 1:1000), and the next 6 rows were separate for positive serum (f_r = 1:250, 1:1000,1:4000, 1:16000, 1:64000, 1:256000), 100 mm^3 each well, and then the strips were kept for 1 h at room temperature.

4. The goat anti-rabbit IgG marked with horseradish peroxidase (HRP) was added, 100 mm^3 each well, and then the solution was kept for 1 h at room temperature.

5. TMB, a kind of color development reagent, was added and then the solution was kept for 15 min at room temperature.

6. H_2SO_4 (2 mol dm^{-3}) was added to stop the reaction and the absorbance at l = 450 nm (OD_{450}) was determined.

In each previous step, the solution was washed with PBST solution 3 times, each time for 3 min. A diagram, OD_{450} as ordinate, minus logarithm of dilution times of antibody as abscissa, was drawn. With P/N (positive/negative) $2.1, then it is considered to be the titer of antibody.

ELISA procedure

After blocking the plates with MPA-OVA, sample (50 μL) or standard was dispensed into the appropriate well using a positive displacement pipette. Rabbit anti-MPA antibody (50 μL) was added to each well and plates were incubated for at least 2 h. Each plate was washed before the addition of goat anti-rabbit HRP-IgG (100 μL per well). After another 2 h of incubation, followed by washing, substrate solution (100 μL per well) was added. Color development proceeded in the dark for about 20 min. The assay was stopped by the addition of 100 μL of 1.25 M H_2SO_4 per well and the absorbance read at 450 nm on a Multiscan MK_3 Luminometer. The zero standard was assayed in quadruplicate, and all other standards and samples were assayed in duplicate. The 4-parameter sigmoidal standard curve would be obtained based on the previously described data.

The assay of cross reactivity (CR)

There are several structural analogs of MPA: estradiol, megestrol acetate, melengestrol acetate, chlormadinone acetate, epitestosterone (ETS), nandrolon (17β-NT), 17α-hydroxyprodesterone, 17α-methyl-testosterone (MTS), testosterone 17-propionate (PTS), cortisol, 17β-estradiol 3-benzoate (BES), progesterone (PG), dexamethasone, prednisolone, pregnenolone, and deoxycorticosterone. These standard compounds were prepared with the same buffer to MPA at different concentrations, which acted as competitive substances to antibody. All operations were carried out according to the ic-ELISA process, and 50% inhibit concentration (IC_{50}) values were calculated based on the sigmoidal curves, respectively: CR(%) = (IC_{50} of MPA)/(IC_{50} of anabolic steroid analog) × 100%.

Table 1—Cross reactivity of medroxyprogesterone acetate (MPA) antisera with anabolic steroids

Anabolic steroid analog	Cross reactivity (%)
Medroxyprogesterone	96
Megestrol acetate	48
Melengestrol acetate	31
Chlormadinone acetate	43
Estradiol	21
17β-estradiol 3-benzoate	16
Progesterone(PG)	18
Pregnenolone	8
Epitestosterone	<0.1
Nandrolon	<0.1
17α-methyltestosterone	<0.1
Testosterone 17-propionate	<0.1
Deoxycorticosterone	<0.1
17α-Hydroxyprodesterone	<0.1
Prednisolone	<0.1
Cortisol	<0.1
Dexamethasone	<0.1

Sample pretreatment protocol

Sample preparation. Healthy pigs were raised on feeds without using any anabolic steroids on our school laboratory farm. When they were slaughtered, muscle tissues were taken out separately, and fat, skin, and bones were discards. Each pork muscle (100 to 200 g) was cut into pieces and treated with a household mixing machine and then preserved by keeping it at –20 °C.

Homogenization. Pork tissue samples (5.0 ± 0.1 g) were transferred to a centrifuge tube (50 mL), and then acetic acid buffer (10 mL) was added. The solution was homogenized at 10000 rpm twice, each time for 20 s using a homogenizer.

In the preparation of spiked samples, homogenized standard solution was added into a centrifugal tube, and then the procedure was as described previously.

Extraction and clean-up. Methanol (10 mL) was added into the solution after enzymolysis. It was mixed by a vortex oscillator for 1 min, extracted by immersing the tube into an ultrasonic bath for 5 min at room temperature, and centrifuged at 2500 × *g* for 10 min. The supernatant was moved to another tube, mixed with tert-butyl methyl ether (15 mL) by oscillator and then centrifuged at 2000 × *g* for 3 min. The bottom layer was reextracted with tert-butyl methyl ether (20 mL, 15 mL) twice. After collecting the ether layers, they were transferred into a rotary evaporator flask and dried at 40 °C. The residue was dissolved in 0.5 mL methanol, and 5 mL water was added.

The solution in the flask was loaded into a C_{18} solid-phase extraction cartridge (500 mg/3 mL), which had previously been primed with methanol (3 mL) and water (3 mL). The flask was washed with 5 mL methanol-water (10%, v/v) and the solution was transferred into the cartridge. The cartridge was rinsed with 5 mL methanol (10%, v/v) and 5 mL water, dried under vacuum for at least 1 min and finally eluted with 6 mL methanol at a flow rate of 1 mL/min. The eluates were dried at 40 °C under a stream of nitrogen and vacuum.

The residues were dissolved in 0.5 mL acetonitrile-water (50%, v/v). The supernatant was analyzed by ELISA and LC/MS/MS.

Table 2—Coefficient of variation between microplates, batches, and in batches (n = 6)

	Standard concentration of medroxyprogesterone acetate (MPA) (ng/g)						
	Blank	0.0	0.1	0.3	0.9	2.7	8.1
%C.V. (between microplates, n = 6)	7.4	4.7	5.6	5.8	2.6	3.8	2.9
%C.V. (between batches, n = 6)	9.5	6.9	8.2	7.9	6.5	5.7	6.1
%C.V. (in batches, n = 6)	13.2	13.7	11.5	12.1	7.8	8.5	10.3

Recovery

Recoveries were calculated based on optical density (OD) values of samples with different additional concentrations, respectively. OD values were interpolated from the standard curve using a computerized technique for automating calculation.

Results and Discussion

Analysis of conjugate

Although the molecular masses of MPA-BSA and BSA are close, the conjugate band still showed the tailing phenomenon and it was lower than that of BSA. Analysis by electrophoresis indicated that the 2 methods of coupling were both successful.

According to the scanning illustration, the maximum wavelength of MPA was 247 nm. The molar extinction coefficient of MPA ({ϵ}) was found to be 15015 by drawing a diagram.

Calculation coupling rates of conjugate

Under 2 different concentrations, 26 μg cm^{-3} and 65 μg cm^{-3}, the absorbances of BSA and MPA-BSA at λ = 247 nm can be known and the coupling rates can also be calculated to be 14 and 13.

Under 2 different concentrations, the absorbances of BSA and MPA-BSA at λ = 247 nm can be known and the coupling rates are 19 and 20.

Titer of antibodies

The highest titer of antiserum could come to 2.6×10^5 according to Figure 1.

ELISA procedure

The optimized ic-ELISA standard curve is shown in Figure 2. The maximum and minimum values of OD were 1.05 and 0.16, respectively, IC_{50} (concentration causing 50% inhibition of binding) was 1.22 ng/g. LOD (the corresponding concentration causing 90% inhibition of binding) was 0.096 ng/g, assay range (the corresponding concentration of 20% to 80% inhibition of binding) was 0.24 to 9.45 ng/g. The dashed lines in Figure 2 represent the concentration of 90%, 80%, 50%, and 20% inhibition of binding, respectively.

Assay of cross reactivity (CR)

The cross reactivity of MPA antisera with anabolic steroid analogs was carried out by ic-ELISA. CR of medroxyprogesterone was 96%. CR of epitestosterone, nandrolon, 17α-methyltestosterone, testosterone 17-propionate, deoxycorticosterone, 17α-hydroxyprodesterone, prednisolone, Cortisol, and dexamethasone were less than 0.1%. CR of 17β-estradiol 3-benzoate, progesterone (PG), and pregnenolone were less than 20%. CR of megestrol acetate, melengestrol acetate, chlormadinone acetate, and estradiol were more than 20%.The results are presented in Table1.

Precision and accuracy

To assess the precision and accuracy of the assay, spiked MPA in muscle tissues at 6 concentrations, corresponding to 0, 0.1, 0.3, 0.9, 2.7, and 8.1 ng/g were studied, as shown in Table 2. Coefficient of variation between microplates was 2.6% to 7.4%. Coefficient of variation between batches was 5.7% to 9.5%. Coefficient of variation between batches was 7.8% to 13.7%. All of the coefficients of variation were less than 15%.

Recovery

Recovery of added MPA in the pork tissues was 72% to 91% for concentrations within the range of 1 to 3 ng/g of ELISA, as shown in Table 3.

To evaluate the performance of the LC/MS/MS method, pork blank samples were fortified at 1.0 and 2.0 μg/kg. Table 4 shows the results of the fortification tests of each steroid in pork tissues. At fortification concentration of 1.0 μg/kg, the average recovery of the MPA was 64%. The coefficient of variation (CV) was 15% at the 1.0-μg/kg level. At fortification concentration of 2.0 μg/kg, the average recovery of MPA was 76% and CV% was 7.9%.

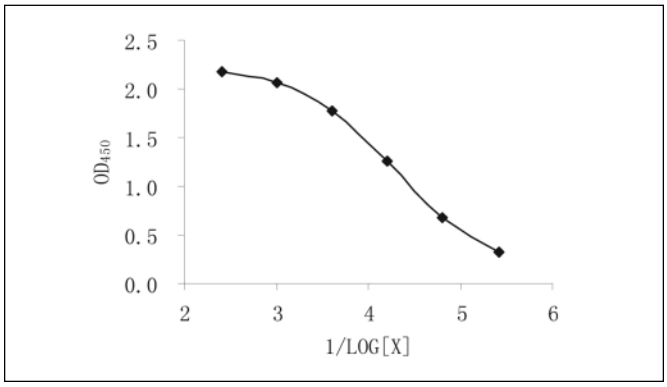

Figure 1—Determination of the titer of the antiserum by indirect enzyme-linked immunosorbent assay (ELISA)

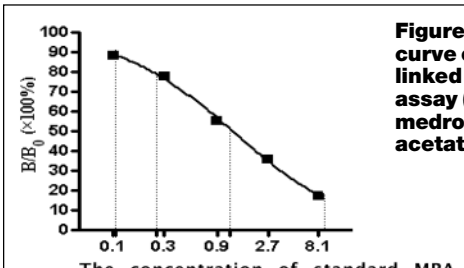

Figure 2—Calibration curve of ic–enzyme-linked immunosorbent assay (ic-ELISA) for medroxyprogesterone acetate (MPA)

Comparison with LC/MS/MS

To evaluate the performance of the methods, pork blank samples were fortified at the 1.0- and 2.0-μg/kg level. Six replicates for each concentration were determined by ELISA and HPLC/MS/MS. The result are shown in Table 5 and Figure 3 to 7. Ion pair for quantification of MPA was 345.2/123.1, and linear equation of MPA of LC/MS/MS: $Y = 6.68 \times 10^3 X + 6.63 \times 10^2$, $r = 0.9998$.

LOD and LOQ

By determining the standard errors of blank samples, the limits of detection (LOD), which were defined as 3 times the standard errors of blank samples, were obtained. Different matrixes did not affect the LOD of the analyte. The LOD of the method for the MPA was 0.034 μg/kg. The limits of quantification (LOQ) of the method were determined according to the formula S/N = 10 and were 0.085 μg/kg.

Conclusions

A ic-ELISA detection was developed and validated for the determination of MPA concentrations in animal muscle tissues. This assay was verified by HPLS/MS/MS. The 2 analytical methods can be applied to measure MPA at subnanogram levels, and practicable for monitoring MPA residues in edible foods.

Table 3—Average recoveries of pork muscle samples as analyzed by ic–enzyme-linked immunosorbent assay (ELISA)

Nr of pork sample	Added concentration of medroxyprogesterone acetate (MPA) (ng/g)	Average value	Coefficient of variation (C.V.%)	Rate of recovery(%)
1	1	0.75	13.5	75
	3	2.57	9.3	86
2	1	0.72	12.4	72
	3	2.72	8.4	91
3	1	0.81	12.1	81
	3	2.68	8.7	89

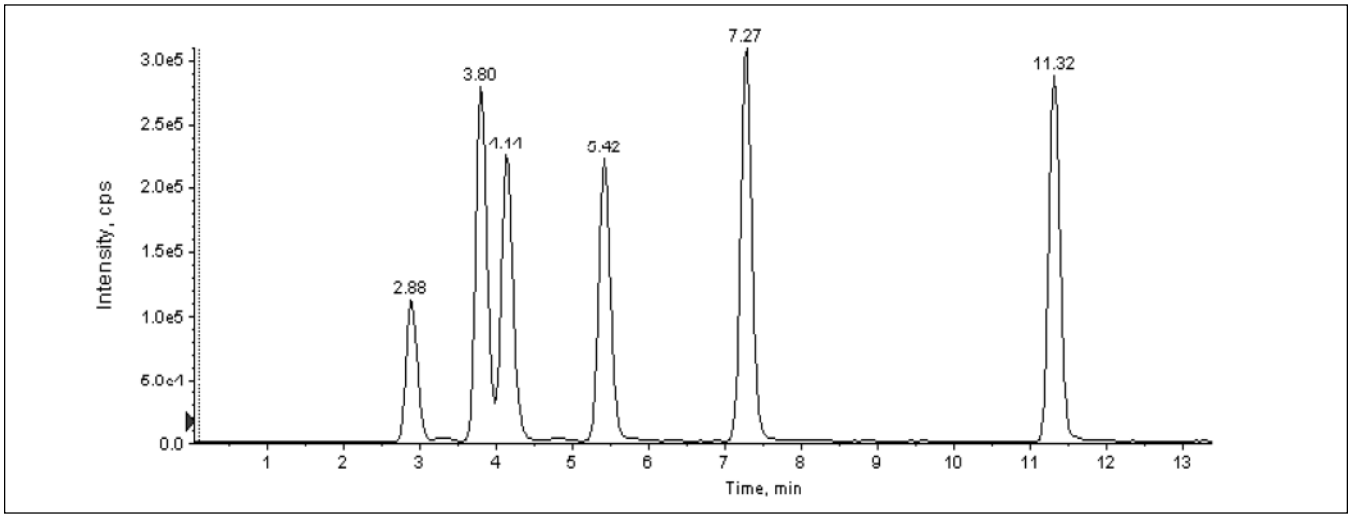

Figure 3—Total ion chromatography of standard androgens and gestagens in LC/MS/MS

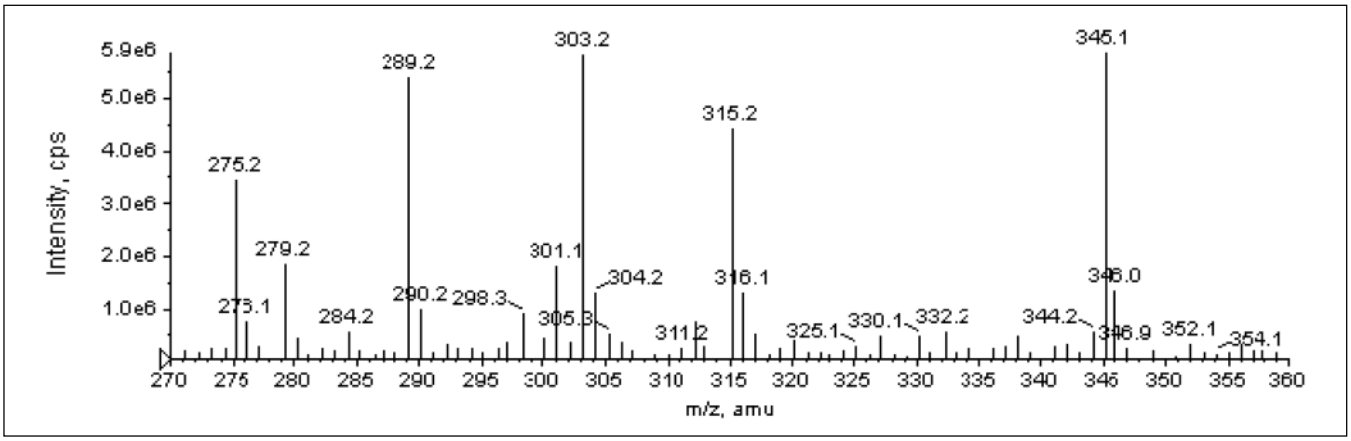

Figure 4—Full scan positive Electronic Spray Ion Q1 scan of nandrolon (17β-NT), 17α-methyl-testosterone (MTS), epitestosterone (ETS), medroxyprogesterone acetate (MPA), progesterone (PG), and testosterone 17-propionate (PTS)

Table 4—Recoveries and precision of the liquid chromatography tandem mass spectrometry (LC/MS/MS) method (n = 6)

Spiked sample (μg/kg)		Nandrolon (17β-NT)	17α-Methyl-testosterone (MTS)	Epitestosterone (ETS)	Medroxy-progesterone acetate (MPA)	Progesterone (PG)	Testosterone 17-propionate (PTS)
1.0	Mean ± SD (μg/kg)	0.71 ± 0.15	0.74 ± 0.25	0.69 ± 0.24	0.64 ± 0.16	0.65 ± 0.21	0.67 ± 0.18
	Recovery (%)	71	74	69	64	65	67
	C.V. (%)	19.6	20.2	19.2	15	19.0	17.7
2.0	Mean ± SD (μg/kg)	1.69 ± 0.28	1.54 ± 0.24	1.70 ± 0.23	1.52 ± 0.14	1.44 ± 0.17	1.43 ± 0.23
	Recovery (%)	84.5	77	85	76	72	71.5
	C.V. (%)	11.2	9.9	12.7	7.9	12.9	11.8

Table 5—Comparative results between enzyme-linked immunosorbent assay (ELISA) and liquid chromatography tandem mass spectrometry (LC/MS/MS) for medroxyprogesterone acetate (MPA) in pork muscle tissues

Method	ELISA		LC/MS/MS	
Added (ng/g)	1.0	2.0	1.0	2.0
Determined (ng/g)	0.65	1.54	0.64	1.52
C.V.(%)	8.7	12.3	9.8	13.5
Nr of determined samples	6	6	6	6

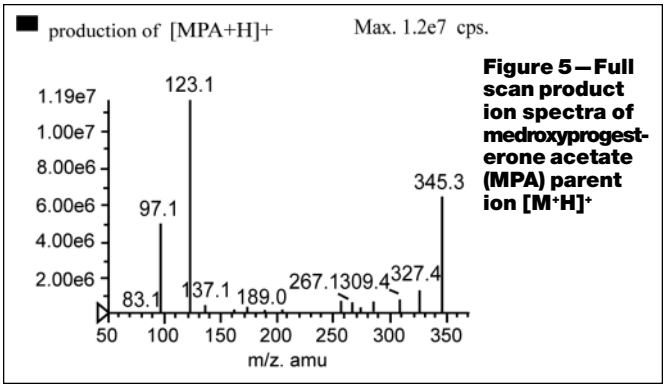

Figure 5—Full scan product ion spectra of medroxyprogesterone acetate (MPA) parent ion $[M^+H]^+$

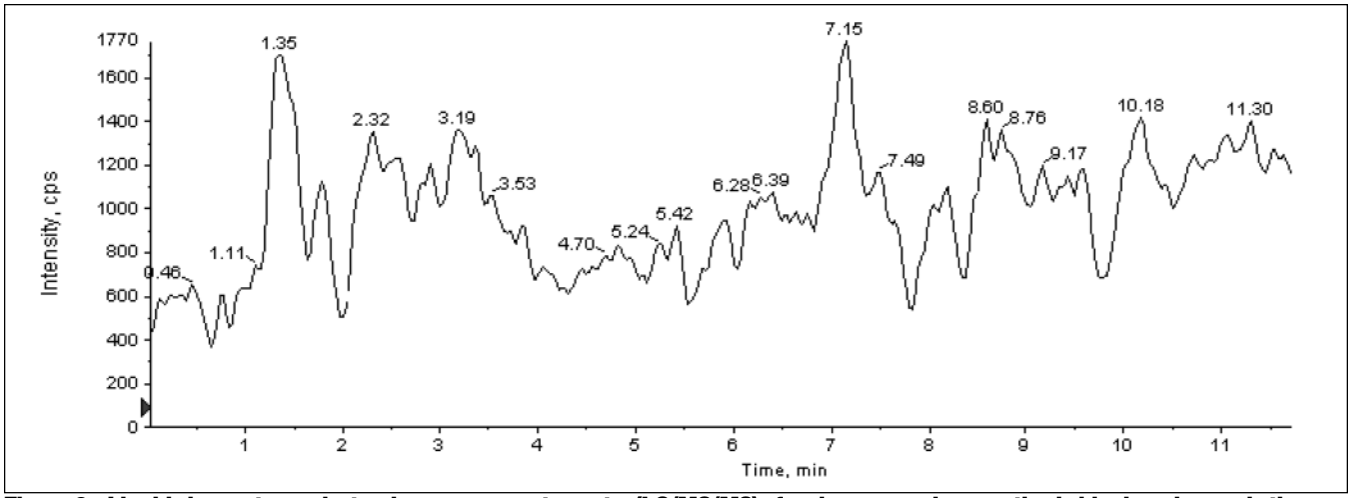

Figure 6—Liquid chromatography tandem mass spectrometry (LC/MS/MS) of androgens and progestins in blank pork muscle tissues

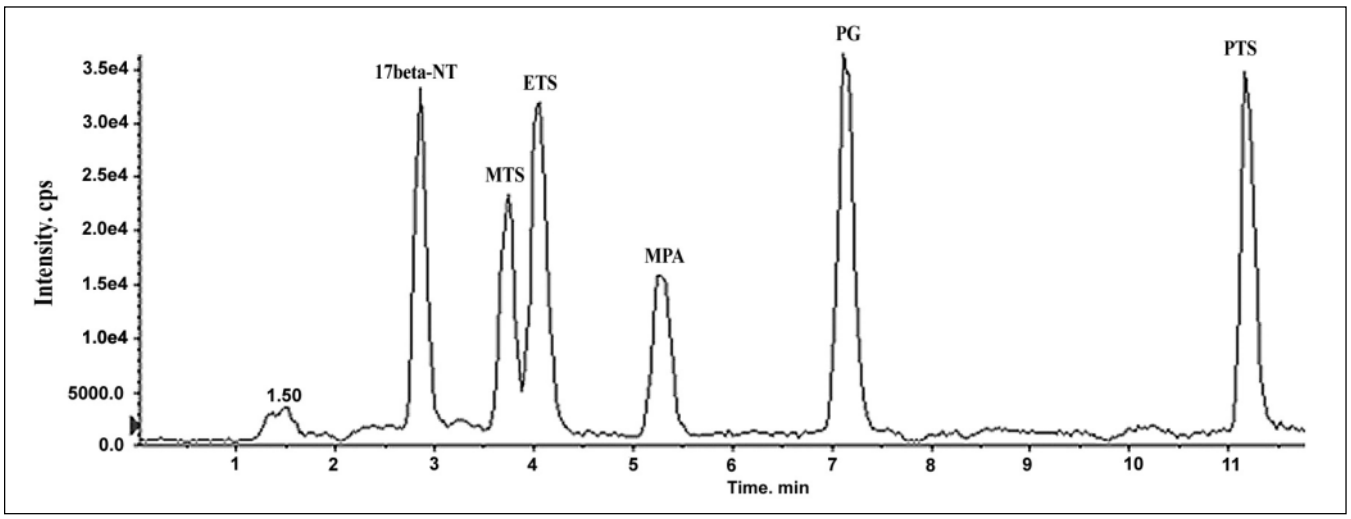

Figure 7—LC/MS/MS spectrometry of androgens and gestagens in spiked pork at 1.0 μg/kg

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References

Bernard FE. 1971. The preparation of antigenic hapten-carrier conjugate: a survey. Princip Meth 70:85–103.
Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–54.
Choi MH, Kim KR, Chung BC. 2000. Simultaneous determination of urinary androgen glucuronides by high-temperature gas chromatography-mass spectrometry with selected ion monitoring. Steroids 65:54–9.
Daeseleire E, De Guesquiere A, Van Peteghem C. 1991. Derivatization and gas chromatographic-mass spectrometric detection of anabolic steroid residues isolated from edible muscle tissues. J Chromatogr 562:673–9.
Draisci R, Palleschi L, Ferretti E, Marchiafava C, Lucentini L, Cammarata P. 1998. Quantification of 17β-estradiol residues in bovine serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization. Analyst 123:2605–9.
Lewis LK, Elder PA, Barrell GK. 1992. An enzyme linked immunosorbent assay (ELISA) for plasma medroxyprogesterone acetate (MPA). J Steroid Biochem Molec Biol 42:179–83.
Philippe AG, Marie CS, Richard HS. 1999. Quantitative analysis of clenbuterol in meat products liquid chromatography-electrospray ionisation tandem mass spectrometry. J Chromatogr B 736:209–19.
Rambaud L, Bichon E, Cesbron N, André F, Bizec BL. 2005. Study of 17β-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. Anal Chim Acta 532:165–76.
Rapp M, Meyer HH. 1989. Determination of hormone contaminants in milk replacers by high-performance liquid chromatography and immunoassay. J Chromatogr 489:181–9.
Shen GX, Zhou RL. 1998. Techniques of modern immunological experiment. Wuhan, China: Press of Scientific Technology in Hubei. p 157–8.
Stolker AA, Zoontjes PW, Schwillens PL, Kootstra PR, van Ginkel LA, Stephany RW, Brinkman UA. 2002. Determination of acetyl gestagenic steroids in kidney fat by automated supercritical fluid extraction and liquid chromatography ion-trap mass spectrometry. Analyst 127:748–54.
Wang CQ. 1997. Basis of molecular immunology. Beijing, China: Press of Beijing Univ.. p 13–5.
Wang JZ, Fan M. 2000. Handbook of protein technology. Beijing, China: Press of Science. p 111–22.
Yang LG, Hu SC, Wei PH, Guo AZ. 1998. Techniques of enzyme immunoassay. Nanjing, China: Press of Nanjing Univ. p 189–84.
Yu NH, Ho EN, Leung DK, Wan TS. 2005. Screening of anabolic steroids in horse urine by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 37:1031–8.
Zhou XW, Hu XQ. 2003. Instrumental analysis and experimental technology of biochemistry. Beijing, China: Press of Chemistry Industry. p 271–2.