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Analytical Methods

Development of a sensitive heterologous ELISA method for analysis of acetylgestagen residues in animal fat

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Abstract

A new heterologous ELISA method for detecting acetylgestagen multi-residues in animal fat was developed. An antibody generated has high cross-reactivity with relative progestogens (up to 50%) and no cross-reactivity with other steroids (<0.1%) in homologous ELISA. Three heterologous immunoassay were developed and one of them improved not only sensitivity but also the class-selectivity compared with the homologous assay against these progestogens. The IC_{50} for four acetylgestagens, chlormadinone acetate (CMA), 17α-hydroxyprogesterone acetate (HPA), megestrol acetate (MEGA) and medroxyprogesterone acetate (MPA) were, 4.5, 2.5, 2.9 and 1.8 μg/L, respectively. The cross-reactivity for CMA, HPA and MEGA were 40%, 72% and 62% when they were compared with MPA. Progestogens recoveries from spiked swine fat averaged between 61% and 78%. Results obtained from LC/MS/MS method showed the heterologous ELISA method developed was reliable and suitable for rapid screening the four progestogens residues in fat tissues.

Keywords: Acetylgestagen; Multi-residues; Heterologous ELISA; Fat

1. Introduction

Some synthetic steroidal hormones have been used as growth promoters in animal breeding. The potential harm of hormone residues in food and environment has been well documented (Barel-Cohen et al., 2006; Hooijerink, van Bennekom, & Nielen, 2003). Synthetic progestogens are able to stimulate other anabolic steroids like estradiol and increase the efficiency of animal production. The use of anabolic steroids has been banned or strictly limited in animal food breeding in many countries including China (The Commission of the European Communities, 2002; The Farming and Veterinarian Bureau Ministry of agriculture of China, 2003). If these anabolic steroids are used in cocktail or alternately, the residue amount of one steroid will decrease and need more sensitive method to detect. Some acetylgestagens, for example, chlormadinone acetate (CMA), 17α-hydroxyprogesterone acetate (HPA), megestrol acetate (MEGA), melengestrol acetate (MGA), and medroxyprogesterone acetate (MPA) are often used in livestock breeding for therapy and growth promotion (The Joint FAO/WHO Expert Committee on Food Additives, 1981). Many physicochemical methods, including GC–MS, GC–MS/MS and LC–MS/MS, have been reported for determining these acetylgestagen residues in animal fat (Hooijerink et al., 2003; Impens, Courtheyn, Wasch, & De Brabander, 2003; Impens, De Wasch, Cornelis, & De Brabander, 2002; Peng, Xu, Jin, Chu, & Wang, 2006; Peng, Xu, & Jin, 2006). But these methods are time-consuming and require sophisticated equipment.

Immunoassays can be employed as a simple, rapid, and cost-effective alternative to those traditional methods in

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cases where high-throughput screening is needed. For the determination of low-molecular-weight haptens, competitive ELISA formats are widely used. They can be divided in general into homologous and heterologous formats. In homologous formats the same hapten is used for immunization and assay purposes, whereas in heterologous formats the immunizing hapten and the competitor hapten (ELISA hapten) differ in their molecular structures (Holthues, Pfeifer-Fukumura, Sound, & Baumann, 2005). Heterologous immunoassay has been successfully applied in nature gestagen analysis, for example, progesterone (Basu, Shrivastav, & Maitra, 2006). But there has been no report on heterologous ELISA method for detecting acetylgestagen. Although ELISA method against MPA in sera has been developed (Lewis, Elder, & Barrell, 1992), few reports on the detection acetylgestagen residues by ELISA in animal tissues has been made. Moreover, compared with time-resolved fluorescence immunoassay (Tr-FIA) and capillary electrophoresis immunoassay (CEIA) (Fiet, Giton, Auzerie, & Galons, 2002; Peng, Huo, Liu, Chu, & Xu, 2007), ELISA method holds obvious advantage of low cost. In this study we developed a new heterologous ELISA method for detecting acetylgestagen residues in animal fat tissue.

2. Materials and methods

2.1. Chemicals and instruments

Reagents were obtained from the following sources: tributylamine and isobutylchlorofomate, bovine serum albumin (BSA) and ovalbumin (OVA), MPA, MGA and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma–Aldrich (Shanghai, China); CMA, MEGA, HPA, hydrocortisol, progesterone and hydrocortisone were from Baida Pharmaceutical (Taizhou, China); horseradish peroxidase labeled goat anti-rabbit IgG antibody (HRP-anti-IgG) were from Rockland Immunochemicals (Philadelphia, USA); acetonitrile, ethyl acetate and hexane (HPLC grade) were from Merk (Shanghai, China). All other reagents were of analytical grade. The ELISA was carried out in 96-well polystyrene microtiter plates (Stripwell plate 2592, Costar, Shanghai, China).

Ultraviolet spectrograms were screened with a 2128-PCS spectrophotometer (Unic, Shanghai, China). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a UNITY-400 spectrometer (400 Hz, FT mode, Varian, Shanghai, China) for solutions in deuteriochlorform ($CDCl_3$). Chemical shifts are given relative to tetramethylsilane (TMS). Electrospray ionization (ESI) mass spectra were obtained on a Platform ZMD 4000 LC–MS (Waters, Shanghai, China). Well absorbencies were read with a MK3 microplate reader (Thermo, Shanghai, China).

2.2. Buffers and solutions

The buffers used regularly were (a) coating buffer; 50 mmol/L carbonate buffer (pH 9.5), (b) phosphate buffered saline (PBS), 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl, (c) dilution buffer, PBS containing 0.1% (*w*/*v*) gelatin, (d) washing buffer (PBST), 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl and 0.05% (*v*/*v*) Tween 20, (e) blocking buffer, PBS containing 1% (*w*/*v*) OVA, and (f) substrate solution; 50 mmol/L sodium citrate buffer (pH 5.0) containing 0.01% (*w*/*v*) TMB and 0.005% (*v*/*v*) H_2O_2. And borate buffer is 0.2 mol/L boric acid–sodium borate, pH 8.7.

2.3. Hapten synthesis

2.3.1. Synthesis of medroxyprogesterone acetate-3-O-carboxylmethyl-oxime (3-CMO-MPA)

To a solution of MPA (386 mg, 1 mmol) in methanol was added aminooxyacetic acid hemihydrochloride (110 mg, 1 mmol) and sodium acetate (136 mg, 1 mmol) (Fiet et al., 2002). The solution was stirred for 24 h at room temperature. After concentration in vacuum, the solid was dispersed with water (5 ml) and acidified to pH 2 with 1 M HCl. The 3-CMO-MPA was extracted with ethyl acetate (3×20 ml). The organic layer was dried on Na_2SO_4 and concentrated in vacuum to obtain 3-CMO-MPA as a white solid which is a 70:30 mixture of *E*–*Z* stereoisomers. ESI-MS, *m*/*z* (relative intensity) 459 (100, $[M-1]^-$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.66 (s, 3 H, 18-CH_3); 1.06 and 1.10 (2s, 3H, E and Z 19-CH_3); 2.03 (s, 3H, CH_3CO); 2.10 (s, 3H, CH_3COO); 4.60 and 4.63 (2s, 2H, CH_2, Z 30% and E 70% CMO); 6.50 and 7.26 (2s, 1H, E and Z 4-H).

2.3.2. Synthesis of megestrol acetate-3-O-carboxylmethyl-oxime (3-CMO-MEGA)

The synthesis of 3-CMO-MEGA was made from MEGA in the same way as the synthesis of 3-CMO-MPA. The synthesized compound was a 70:30 mixture of *E*–*Z* stereoisomers. ESI-MS, *m*/*z* (relative intensity) 457 (100, $[M-1]^-$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.70 (s, 3H, 18-CH_3); 0.95 and 1.00 (2s, 3H, E and Z 19-CH_3); 2.05 (s, 3H, CH_3CO); 2.09 (s, 3H, CH_3COO); 4.63 and 4.67 (2s, 2H, CH_2, Z 30% and E 70% CMO); 5.71, 6.00 (2d, 1H, E and Z 7-H); 6.61 and 7.26 (2s, 1H, E and Z 4-H).

2.3.3. Synthesis of chlormadinone acetate 3-O-carboxylmethyl-oxime (3-CMO-CMA)

The synthesis of 3-CMO-CMA was made from CMA in the same way as the synthesis of 3-CMO-MPA. The synthesized compound was a 70:30 mixture of *E*–*Z* stereoisomers. ESI-MS, *m*/*z* (relative intensity) 477 (100, $[M-1]^-$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.70 (s, 3H, 18-CH_3); 1.00 and 1.05 (2s, 3H, E and Z 19-CH_3); 2.05 (s, 3H, CH_3CO); 2.09 (s, 3H, CH_3COO); 4.66 and 4.70 (2s, 2H, CH_2, Z 30% and E 70% CMO); 6.05, 6.15, (2d, 1H, E and Z 7-H); 6.50 and 7.11 (2s, 1H, E and Z 4-H).

2.3.4. Synthesis of 17α-hydroxyprogesterone acetate-3-O-carboxylmethyl-oxime (3-CMO-HPA)

The synthesis of 3-CMO-HPA was made from HPA in the same way as the synthesis of 3-CMO-MPA. The synthesized compound was a 70:30 mixture of *E–Z* stereoisomers. ESI-MS, *m/z* (relative intensity) 445 (100, $[M-1]^-$); ^{1}H NMR (400MHz, $CDCl_3$): δ 0.53 (s, 3H, 18-CH_3); 1.06 and 1.10 (2s, 3H, E and Z 19-CH_3); 2.03 (s, 3H, CH_3CO); 2.10 (s, 3H, CH_3COO); 4.60 and 4.62 (2s, 2H, CH_2, Z 30% and E 70% CMO); 6.05, 6.15, (2d, 1H, *E* and *Z* 7-H); 6.50 and 7.11 (2s, 1H, *E* and *Z* 4-H).

2.4. Immunochemistry

2.4.1. Preparation of conjugates of haptens and proteins

The conjugates of haptens and proteins were prepared by the mix anhydride method (Oubina, Barcelo, & Marco, 1999). Hapten (10 mg, 26 μmol) (3-CMO-MPA, or 3-CMO-CMA, 3-CMO-MEGA, or 3-CMO-HPA) was reacted with tributylamin (12 μl, 49.5 μmol) and isobutylchlorofomate (9 μl, 54 μmol) in *N*,*N*-dimethylformate (DMF, 0.4 mL) for 1 h at 10 °C. The solution containing the activated hapten was added dropwise to 60 mg BSA (or OVA) solution in 0.2 M borate buffer (6 mL) and DMF (2 mL), and the reaction was undertaken with stirring for 2 h at 10 °C. The conjugates were purified by dialysis against 0.5 mM PBS (5 × 2 L) and ultra pure water (3 × 2 L) and stored freeze-dried at −50 °C. Conjugate formations were screened spectrophotometrically. Molar ratios of approximately 11.4, 7.2, 8.8, 7.4, and 5.3 were obtained for 3-CMO-MPA-BSA, 3-CMO-MPA-OVA, 3-CMO-CMA-OVA, 3-CMO-MEGA-OVA and 3-CMO-HPA-OVA conjugates, respectively.

2.4.2. Preparation of polyclonal antibody

The immunization protocol was performed on female New Zealand white rabbits weighing 1.5–2.0 kg as previously described (Sheth & Sporns, 1991). The rabbits were first injected with 0.5 mg immunogen (3-CMO-MPA-BSA) in 1.0 ml oil emulsion consisted of sterile physiological saline and Freund's complete adjuvant (FCA) (1:1, *v/v*) subcutaneously and gluteal. After four weeks, booster injection was undertaken with identical oil emulsion except the Freund's incomplete adjuvant (FIA) was used rather than FCA. Following 6–8 booster injections at intervals of 2 or 3 weeks, blood was collected from the marginal ear vein and by cardiac puncture one week after last injection. The blood was left for 2 h at room temperature to form blood clot. The serum was separated from the clot by centrifuged and stored at −20 °C in the presence of 50% glycerol and 0.1% NaN_3.

2.4.3. Indirect competitive ELISA

For indirect competitive assays, the concentrations of coating antigens, antibodies and enzyme tracer (HRP-anti-IgG) were optimized by checkerboard titration. Polystyrene 96-well microplates were coated with 100 μL/well of hapten-OVA conjugates in coating buffer at optimized concentrations overnight at 4 °C. After washing three times with PBST, the plates were treated with blocking buffer, sealed and stored for 2 h at 37 °C. The sensitized plates were washed with PBST and patted dry with paper towel. Serial dilutions of the progestogen standards in PBST were added into microplate (50 μL/well), followed by antibody (1/5000 in dilution buffer, 50 μL/well). After 1 h of incubation time at 37 °C, the plates were washed again as before, and a solution of anti-IgG-HRP (1/5000 in dilution buffer) was added (100 μL/well) and incubated for an additional 30 min at 37 °C. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was continued at 37 °C and stopped after 15 min with 2 M H_2SO_4, and the absorbances were read at 450 nm. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentrations.

2.5. Sample extraction and clean-up

Negative swine fat samples were obtained from Food Laboratory, Jiangsu Import & Export Inspection and Quarantine Bureau (China).

A fat sample of 10 g was cut into small pieces. Brought into a funnel with cotton wool, the fat was melted in a microwave oven. Two gram fat was transferred into 50 mL polypropylene centrifuge tube. A series of rendered swine fat samples were prepared. Each sample of 2 g was spiked with 2, 4 or 10 μL of 1.0 μg/mL MPA standard solution in methanol to obtain the tissues equivalent concentration of 1.0, 2.0 and 5.0 μg/kg MPA. Another series of blank samples of 2 g were spiked with 4, 8 or 20 μL of 1.0 μg/mL CMA, MEGA or HPA standard solution in methanol to obtain the tissues equivalent concentration of 2.0, 4.0 and 10.0 μg/kg CMA, MEGA or HPA.

The 5 mL acetonitrile was added to 2 g of spiked sample of fat. The mixture was shaken vigorously at 60 °C for 15 min and then cooled in running tap water. The mixture was then centrifuged for (2000*g*, 10 min) and the supernatant was decanted. The extraction by acetonitrile was repeated on the precipitated fat portion. The supernatants were collected and combined. The acetonitrile extract was cooled in a freezer at −40 °C for 45 min and centrifuged at 4 °C (2000*g*, 5 min). Then the supernatant was decanted into a flask and evaporated using a rotavapor. The residue was dissolved in 2.0 mL hexane, and passed through a CN-SPE cartridge (500 mg/3 mL, Bondelute, Varian) conditioned with 3 mL ethyl acetate and 5 mL hexane. The cartridge was washed with 5 mL hexane and then eluted with 3 mL ethyl acetate-hexane (90:10, *v/v*). The elution was dried and reconstituted in 1 mL PBST buffer by vortex for ELISA analysis.

3. Results and discussion

3.1. Hapten selection and synthesis

In this study we prepared four complete antigens to develop four ELISA methods and to select one by applying

different complete antigen to coat the microplate. One of the four methods (3-CMO-MPA-OVA was coating antigen) is homologous ELISA and three others are heterologous. In the present study, we adopted the facile synthesis method to obtain four haptens. The synthesis is a simple process and can be carried out at room temperature. Four coating antigens were obtained through conjugating the four haptens with carrier protein OVA.

3.2. Characterization of the antibody and homologous ELISA

The cross-reactivity (CR) was calculated according to the following equation:

$$\text{Cross-reactivity}\% = \frac{\text{standard } IC_{50}}{\text{cross-reactant } IC_{50}} \times 100$$

where IC_{50} is MPA concentration reducing the ELISA maximum response to 50%. In homologous assay, the antibody showed high cross-reactivity with CMA, HPA and MEGA in the indirect competitive ELISA (Table 1). The structures of the four progestogens are very similar except the changes at C-6 (Table 1). Although other steroids are slightly different in the structure compared with the four steroids, the cross-reactivity is very low and negligible. So the developed homologous ELISA is class-selective and suitable for the four progestogens screening detection.

3.3. Heterologous ELISA methods

The sensitivity of the heterologous ELISA was significantly higher than that of the homologous ELISA when coating antigen was changed from 3-CMO-MPA-OVA to 3-CMO-HPA-OVA. Accordingly the average IC_{50} of MPA, CMA, HPA and MEGA all decreased to 1.8 μg/L, 4.5 μg/L, 2.5 μg/L and 2.9 μg/L, respectively (Table 2). These changes were consistent with the previous works in which heterologous assay also improved the sensitivity of immunoassay (Basu et al., 2006; Holthues, Pfeifer-Fukumura, Sound, & Baumann, 2005; Kim et al., 2003; Onishi et al., 2002; Spinks, Wyatt, Everest, Jackman, & Morgan, 2002). Meanwhile, the cross-reactivity of CMA, HPA and MEGA all increased by 3%, 22% and 12%, respectively, compared with the homologous assay. The increased values were. As a result, the differences of the cross-reactivity of CMA, HPA and MEGA compared with that of MPA reduced in the heterologous assay. It can be claimed that the heterologous ELISA is more class-selective than the homologous. A work on ELISA for sulphonamides also showed similar phenomenon (Spinks et al., 2002). As for two other heterologous assays developed, the sensitivity and cross-reactivity both decreased compared with homologous assay. In this study we found that the sensitivity and class-selectivity of the heterologous assays either increased or decreased depending on the change of coating antigens. It should need further investigation to elucidate what causes this phenomenon.

Table 1
Specificity of the antibody in the homologous ELISA using 3-CMO-MPA-OVA as coating antigen

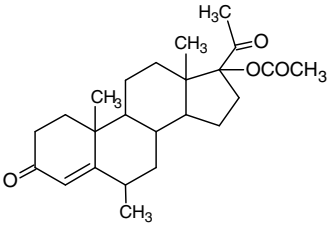
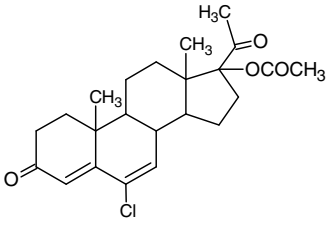
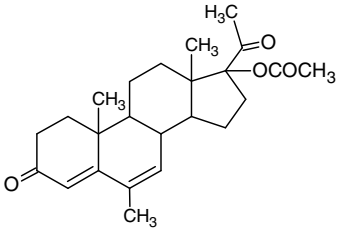
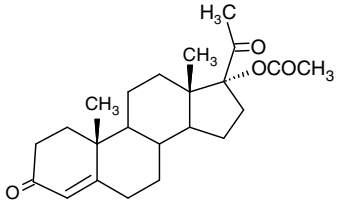
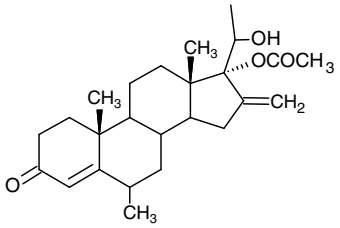
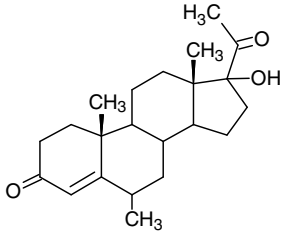
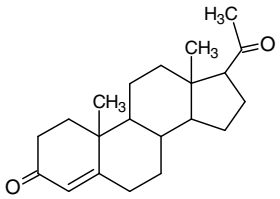
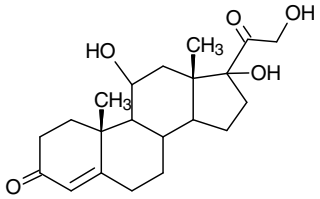
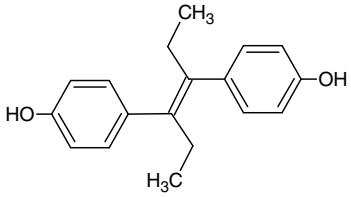
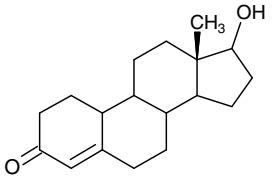
Compound	Structure	IC_{50} (ng/L)[a]	CR (%)[b]
MPA		4.8	100
CMA		13.0	37
MEGA		9.6	50
HPA		9.6	50
MGA		–[c]	<0.1
Medroxyprogesterone		32	15

Table 1 (*continued*)

Compound	Structure	IC_{50} (ng/L)[a]	CR (%)[b]
Progesterone		–	<0.1
Hydrocortisone		–	<0.1
Diethylstilbestrol		–	<0.1
19-Nortestosterone		–	<0.1

[a] MPA concentration reducing the ELISA maximum response to 50%. Data represent the means of six experiments. Standards were diluted in PBST.

[b] Percentage of cross-reactivity = (IC_{50} of MPA/IC_{50} of other compound) × 100. Standards were diluted in PBST.

[c] The IC_{50} cannot be evaluated.

As shown in Fig. 1, the standard curves of four progestagens in the heterologous ELISA (coating antigen: 3-CMO-HPA-OVA) revealed a good linearity ($R^2 > 98\%$) within the range of 0.1–15 μg/L for MPA and that of 0.2–30 μg/L for CMA, MEGA and HPA. These results demonstrated that sensitivity of the heterologous assay developed in this study was higher than or comparable to that of the methods previously reported (Fiet et al., 2002; Peng et al., 2006; Peng et al., 2007).

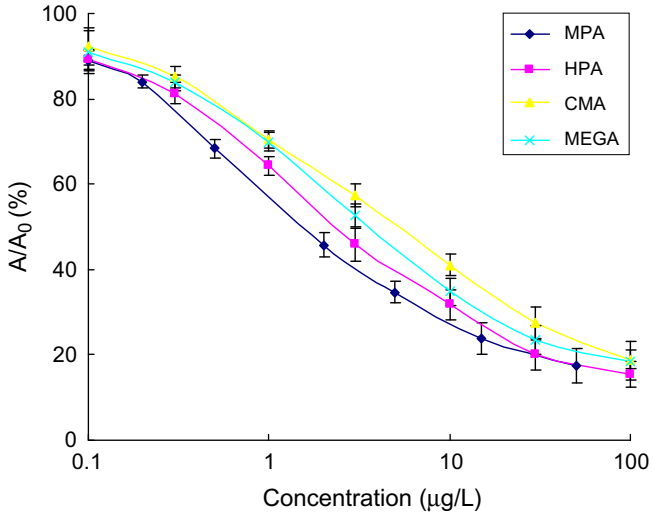


Fig. 1. Standard curves for progestogens ($n = 10$). Conditions: 0.1 μg/mL 3-CMO-HPA-OVA coating antigen.

3.4. Validation

The limits of detection (LOD) were calculated by taking the mean value of 10 blank samples plus 3 times standard deviations of the mean. The LODs were found to be 0.3 μg/kg for MPA, 0.6 μg/kg for CMA, MEGA and HPA in swine fat tissue.

The recovery of the four progestogens was also detected, which could be used to compensate for work-up losses. Considering the differences in sensitivity for four progestogens, the concentration of MPA spiked was lower than the concentration of CMA, MEGA and HPA.

The clean-up for progestogens extraction in fat species usually includes a de-fat step to decrease fat interference (Hageleit et al., 2001; Hooijerink et al., 2003; Impens et al., 2003; Peng et al., 2006). Some hydrophobic solvent such as hexane can be used to de-fat from the acetonitrile solution, but it will probably results in lower recovery because progestogens also are soluble well in hexane. The fat can also be precipitated in the acetonitrile solution at low temperatures, which can largely eliminate the

Table 2
Specificity of the antibody and sensitivity of the ELISA methods using different coating antigen

Coating antigen	MPA		CMA		MEGA		HPA	
	IC_{50} (μg/L)	CR (%)	IC_{50} (μg/L)	CR (%)	IC_{50} (μg/L)	CR (%)	IC_{50} (μg/L)	CR (%)
3-CMO-HPA-OVA	1.8	100	4.5	40	2.9	62	2.5	72
3-CMO-MPA-OVA	4.8	100	13.0	37	9.6	50	9.6	50
3-CMO-MEGA-OVA	8.5	100	26.3	32	20.4	42	20.4	42
3-CMO-CMA-OVA	5.6	100	27.5	20	11.7	48	16.6	34

Table 3
Recovery and repeatability values of MPA obtained in fat samples

Compounds	Spiked concentration (μg/kg)	Average recovery (%) (μg/kg) ($n = 6$)	CV (%)
MPA	1	63	18.5
	2	68	10.3
	5	78	8.8
CMA	2	65	19.1
	4	71	13.5
	10	78	8.7
MEGA	2	62	18.2
	4	71	15.9
	10	77	12.1
HPA	2	62	15.8
	4	75	11.6
	10	73	12.7

Table 4
Progestogen concentration in fat samples collected from local markets ($n = 3$)

Sample	MPA (μg/kg, mean ± SD)	
	ELISA	LC/MS/MS
1	5.9 ± 0.7	4.1 ± 0.3
2	21.5 ± 2.2	15.3 ± 1.2
3	18.7 ± 2.0	14.9 ± 1.1

interference by the fat in the acetonitrile extract. The method has the advantage in reducing the amount of the solvent used in the sample pre-treatment.

In Table 3, it can be found the recovery of the four progestogens averaged between 61% and 78% at three concentration levels of each progestogen. The coefficients of variation (CV) are within 20%. This result demonstrated that the new method developed in this study can be used as a screening method for the four progestogens.

In this investigation we only discussed the detection of the progestogens in swine fat tissues because swine fat is an important animal food in China. However, further research is needed to detect the progestogens in other animal matrixes.

To further demonstrate reliability of the ELISA method for the determination of progestogen residues in swine fat, 10 swine fat samples from local market were analyzed using the ELISA method developed and LC/MS/MS method to validate the developed method. Only MPA was found in three samples (Table 4). The MPA concentrations determined by the ELISA method were 20–30% higher than those determined by LC/MS/MS method. These higher MPA values of ELISA perhaps are attributed to the matrix interferences from other steroids and lipids in the samples.

4. Conclusions

Compared with the homologous immunoassay method developed in this study, a heterologous immunoassay markedly improved the sensitivity and to some extent the class-selectivity for four progestogens. The validation of the method developed showed a good reliability and accuracy. Thus, this heterologous ELISA method is suitable for the rapid screening of the four progestogens residues in animal fat.

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