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A rapid and sensitive enzyme-linked immunosorbent assay (ELISA) method and validation for progestogen multi-residues in feed*

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ABSTRACT

Some progestogens as anabolic steroids can be illegally used for increasing the efficiency of animal food production. The aim of the study was to develop convenient screening method against progestogens in feed. A sensitive enzyme-linked immunosorbent assay (ELISA) method was developed and optimized. The ELISA method is class-specific for four relative progestogens and the IC_{50} of the method was 3.0 μg/l for MPA. A simple feed pre-treatment method was developed for analysis progestogen residues in feed. The limits of detection (LOD) were 2.0 μg/kg for MPA, 5.0 μg/kg for CMA, 4.6 μg/kg for MEGA and 3.9 μg/kg for HPA in the feed. The recovery was between 72.1 and 95.4% and the coefficients of variation (CV) were 7.2 to 15.8%. The results have showed the simple sample pre-treatment method was suitable for progestogen multi-residues rapid analysis in feed.

KEY WORDS: progestogens, multi-residues, ELISA, feed

INTRODUCTION

Synthetic progestogens are able to stimulate other anabolic steroids like oestradiol and increase the efficiency of animal production, e.g., medroxyprogesterone acetate (MPA), melengestrol acetate (MGA), megestrol acetate (MEGA),

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chlormadinone acetate (CMA) and 17α-hydroxyprogesterone acetate (HPA), etc. The use of anabolic steroids has been banned using as a growth promoter for animal food producing in many countries include China. But commercial or other benefits still stimulate people to apply them in cocktail or alternately. Thus a method for progestogen multi-residues analysis is necessary. ELISA method has been demonstrated as a simple, rapid, and cost-effective alternative to those traditional methods in cases where high-throughput and/or on-site screening are needed. Although some specific ELISA method for progestogens, MGA (Hageleit et al., 2001), MPA (Lewis et al., 1992; Peng et al., 2006b) and HPA (Guizhen et al., 1988), and some specimen, serum (Lewis et al., 1992), fat and muscle tissues (Rosen et al., 1994; Hageleit et al., 2001; Peng et al., 2006a) and liver (Peng et al., 2006b) have been reported, no special discussion about analysing these compounds in feed specimen were found. The applied antibody was prepared to develop simple and class-specific ELISA method for analysing some progestogen muti-residues in feed here.

MATERIAL AND METHODS

Chemicals and instruments

Reagents were obtained from the following sources: MPA, MGA and 3,3',5,5'-Tetramethlbenzidine (TMB) (Sigma-Aldrich, Germany); CMA, MEGA, HPA, hydrocortisol, progesterone, hydrocortisone and 19-Nortestosterone (Taizhou Baida Pharmaceutical Co., Ltd., China); horseradish peroxidase - labeled anti-mouse immunoglobulin rabbit IgG ((HRP-anti-IgG) (Rockland Immunochemicals, Inc., USA), acetonitrile, ethyl acetate, tert-butyl methyl ether (TBME) and hexane (Merk, Germany, HPLC grade). Coating antigens 3-CMO-MPA-OVA and anti-MPA antiserum were prepared by our laboratory. All other reagents were of analytical grade. The ELISA was carried out in 96-well polystyrene microtiter plates (Costar sripwell plate 2592, China).

Buffers and solutions

The buffers used regularly were: a. coating buffer; 50 mmol/l carbonate buffer (pH 9.5), b. phosphate buffered saline (0.01 M 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 0.02% (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.

Indirect competitive ELISA

The concentrations of coating antigens, antibodies and enzyme tracer (HRP-anti-IgG) were selected by checkerboard titration. Afterward a set of experimental parameters (incubation time, ionic strength, pH and detergent concentration) was studied. The optimal ELISA method protocol as follows: polystyrene 96-well microplates were coated with 100 µl/well of MPA-OVA conjugates (0.1 µg ml^{-1}) in coating buffer overnight at 4ºC and blocked for 2 h at 37ºC. Serial dilutions of the progestogen standards in PBST (from 0.05 to 50 µgl^{-1}) were added into the sensitized microplate (50 µl/well), followed by the appropriately diluted antiserum (1/5000 in dilution buffer, 50 µl/well). After incubation at RT for 60 min, the plates were washed three times with PBST and patted dry. The appropriately diluted Anti-IgG-HRP (1/5000 in dilution buffer) was then added (100 µl/well) and incubated for 30 min at RT. After a wash procedure again, the substrate solution was added (100 µl/well). Colour development was continued and stopped after 15 min at RT with 2M H_2SO_4, and the absorbances were read at 450 nm by a Thermo MK3 microplate reader (Thermo Electron, China). Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration.

Characterization of the ELISA method

Nine steroid compounds (MPA, CMA, MEGA and HPA, MGA, progesterone, hydrocortisone, diethylstilbestrol and 19-Nortestosterone), which are structurally homogeneous or related, were selected for specific evaluation of the ELISA method.

Stock solutions of 0.1 mg/ml of them in methanol were prepared for serial standard solution. Standard curves for each of these compounds were constructed (0.2 to 200 µg/l in PBST) and their IC_{50} values were determined for cross-reactivity calculation. The cross-reactivity (CR) was calculated according to the following equation:

$$\text{Cross-reactivity \%} = \frac{\text{Standard } IC_{50}}{\text{Cross-reactant }_{IC}} \times 100$$

Sample pre-treatment and validation

The pig and fish feed were ground to powder with a glass pestle. Each sample of 2 g in a tube was spiked with 10, 20 or 40 µl of 1.0 µg/ml MPA standard solution in methanol, respectively. And another series of blank samples of 2 g each were spiked with 20, 40 or 80 µl of 1.0 µg/ml CMA, MEGA or HPA standard solution in methanol. These give the specimen equivalent concentration of 5, 10 and 20 µg/kg

MPA and another one of 10, 20 and 40 μg/kg CMA, MEGA or HPA. Ten ml 20% methanol solution in 0.1 M carbonate buffer was applied into it and homogenized for 1 min at 10,000 rpm. Five ml TBME was then added and mixed by a vortex for 1 min. The tube then stood in a supersonic bath for 10 min till the emulsified phase was broken. The mixture was then centrifuged at 3000 *g* for 10 min and 5 ml TBME phase was decanted. Afterward solvent was dried under nitrogen at 40ºC the residue was reconstituted in 10 ml PBST buffer by vortex for ELISA analysis. The accuracy was evaluated by determining the recovery of spiked standards in 5 parallel samples. The precision was evaluated by determining the relative standard deviation (RSD) 15 samples totally in 3 days (5 samples each day).

RESULTS AND DISSCUSION

Characterization of the ELISA method. Under optimum physico-chemical conditions, the ELISA for MPA reported here has an IC_{50} of 3.0 μg/l, the working range (80-20% of the A_{max}) is placed between 0.3 and 30 μg/l. The A_{max} of the assay was always more than 1.0 units of absorbance.

Results showed (Table 1) that the antibody had high cross-reactivity with CMA (39.5%), MEGA (56.6%) and HPA (62.5%), but no cross-reactivity with other steroids (the IC_{50} of the five steroids can't be evaluated). As screening method of veterinary drug residue, specific for four progestogens will be more useful than one when several veterinary drug need to be inspected.

Table 1. Cross reactivity and LODs of four progestogens in pig feed (n=10)

Parameters	MPA	CMA	MEGA	HPA
IC50, μg/l	3.0	7.6	5.3	4.8
CR, %	100	39.5	56.6	62.5
LOD, μg/l	2.0	5.0	4.6	3.9
R.S.D., %	5.6	7.3	6.4	7.7

Sample pre-treatment. Feed sample was pretreated in alkaline buffer and 20% methanol and then partitioned by tert-butyl methyl ether (TBME), which could reserve most of polar impurity in the buffer and increase the selectivity of TBME extraction. Supersonic could not only break emulsification, but also ensure sufficient extraction and decrease variation of extraction. Nevertheless, the matrix effect was still very notable (Figure 1). After the matrix was diluted by ten times, the effect of it could be decreased mostly. Through matrix addition to the standard solution, the calibration curve could be used to measure the progestogens residue quantitatively.

Two different feed, pig and fish diet, were investigated to compare the difference of different matrix. Figure 2 showed that matrix effect of the pig feed was slightly

lower than that of the fish. Followed by dilution times of the two kinds of matrix increasing, the maximal signal (A_{max}) increased step by step. Figure 2 shows that 10% matrix has been suitable for the ELISA analysis.

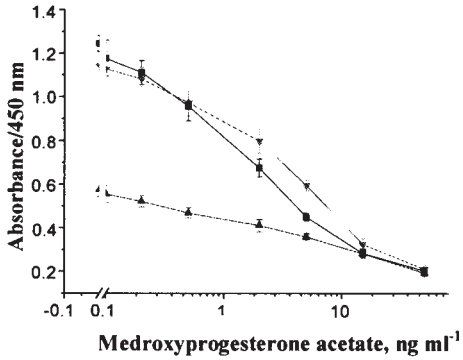

Figure 1. Effect of feed matrix on standard curve of MPA. MPA santandard curve in the PBS (■), in feed matrix diluted 1/10 with PBS (▼), and in neat feed matrix (▲) (n=5)

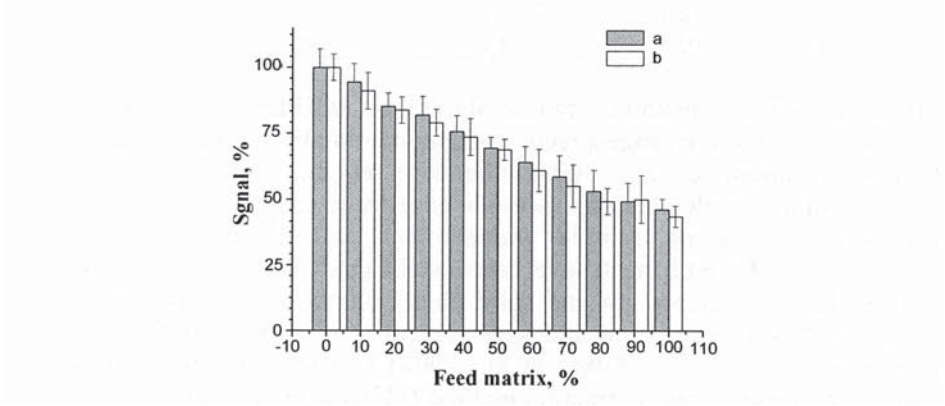

Figure 2. Effect of feed matrix on maximum absorbance of the assay for MPA: a and b represent pig feed and fish feed, respectively. Each point represents the mean value of triplicate measurements (n=5)

Validation. The limits of detection (LOD) were calculated by taking the mean value of 10 blank samples plus 3 times of standard deviations of the mean. The LODs were calculated to be 2.0 μg/kg for MPA, 5.0 μg/kg for CMA, 4.6 μg/kg for MEGA and 3.9 μg/kg for HPA in pig feed after blank pig feed samples were determined (Table 1).

The recoveries of the four progetagens were also determined, which could be used to compensate work-up losses. The results were shown in Table 2. It could be

found the recoveries for them were between 75.0 and 92.1% at three concentration levels for pig feed, and between 72.1 and 95.4% for fish feed. The relative standard deviations (RSD) were 8.2 to 15.8% for pig feed, and 7.2 to 15.5% for fish feed.

Table 2. Inter-day precision and accuracy for progestogens using developed ELISA

Analytes	Spiked level µg/l	Pig feed		Fish feed	
		accuracy recovery, % n=5	inter-day precision RSD, % n=3	accuracy recovery, % n=5	inter-day precision RSD, % n=3
MPA	5	81.4	15.8	72.1	13.4
	10	86.4	11.4	85.3	8.2
	20	88.2	8.2	81.4	9.1
CMA	10	75.0	13.1	80.6	15.5
	20	78.9	8.5	84.9	9.6
	40	77.6	10.7	86.7	9.5
MEGA	10	86.5	15.0	83.8	10.8
	20	90.7	10.9	86.2	7.2
	40	83.1	12.2	85.7	9.0
HPA	10	85.2	10.9	81.1	12.3
	20	89.7	10.3	95.4	8.7
	40	92.1	8.7	85.8	8.4

Because MRLs (maximal residue limits) or MRPLs (minimum required performance limits) the progestogens residue in animal food were as low as 1 to 10 µg/kg (Impens et al., 2003; Giannetti et al., 2005), complex and cost-consuming sample clean-up methods have to be adapted for animal fat and meat specimen. Some sample pretreatment and clean-up methods for progestogens in fat and meat have been established including combination of matrix solid phase dispersion and solid phase extraction (Rosen et al., 1994), accelerated solvent extraction and supercritical fluid extraction for kidney fat (Stolker et al., 2002; Hooijerink et al., 2003) and most of them are solvent extraction and solid phase extraction method (Hageleit et al., 2001; Impens et al., 2002; Giannetti et al., 2005) for kidney fat or meat. Feed containing below 10 µg/kg progestogens is usually ineffective for bringing drug residue in animal tissues, because most of progestogens will be metabolized. Although the simple extraction and standard calibration method for feed developed here have higher coefficients of variation than clean-up method for animal tissues, it still can meet the need for rapid screening progestogens residue in feed.

To further demonstrate reliability of the ELISA method for determination of progestogen residues in feed, 10 pig feed samples and 10 fish samples from local market were analysed using the ELISA method and LC/MS/MS method.

Medroxyprogesterone acetate (MPA) was found in three fish feed samples. The MPA concentrations are presented in Table 3. Compared with the results from LC/MS/MS, the MPA concentrations were consistently higher. This result of overestimating the analyte concentration was probably due to the matrix interference from other steroids and lipids in the ELISA method. The reason need further study in the future.

Table 3. MPA and CMA concentration in feed 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

CONCLUSIONS

In conclusion, the developed ELISA method is specific for four progestogens and suitable for routine qualitative measurement of them in animal feed. The method was sensitive and the sample treatment method was also simple and cost-saving. This strategy is effective in some specimen. However, livestock and aquatic animal feed all have many types and the matrix effects of them will be different for the ELISA method. Thus, the recovery and precision should be investigated when the method is applied to a new kind of feed.

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