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SHORT COMMUNICATION

Rapid Determination of Time-Resolved Fluoroimmunoassay for Medroxyprogesterone Acetate Residues in Pork Tissues and Comparison with Liquid Chromatography and Tandem Mass Spectrometry

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Abstract A competitive time-resolved fluoroimmunoassay (TR-FIA) was developed for the determination of medroxyprogesterone acetate (MPA) residues in pork tissues. The limits of detection (LOD) was determined to be 0.06 ng g^{-1} and the limits of quantification (LOQ) was less than 0.8 ng g^{-1}. The intra-assay variations were below 10% and the interassay variations ranged between 9.7 and 12.7%. The mean recoveries established at six concentration levels varied from 87.3 to 108.3%. The results obtained by the TR-FIA and ELISA showed a good correlation. The established TR-FIA was validated for the determination of incurred pork tissues and confirmed by high-performance liquid chromatography and tandem mass spectrometry (LC-MS-MS). This proposed technique could be applied to routine residue analysis.

Keywords Medroxyprogesterone acetate · Residues · Screening · Immunoassay · Time-resolved fluorometry

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 which can speed up fattening [1]. The presence of traces of MPA in animal tissues poses a potential hazard for consumers. The two methods of determination of anabolic steroid residues in animal muscle tissues are high-performance liquid chromatography and tandem mass spectrometry as well as gas chromatography-mass spectrometry [2–7]. However, these methods required well-equipped laboratories, trained personnel, a high capital expenditure and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining MPA residues contamination in samples.

While residue levels can be detected by instrumental analysis, the large number of samples and analysis labor and times required for thorough monitoring led us to develop an alternative method, suitable for trace element testing by technically competent personnel. Immunoassay is the most suitable testing method for the rapid field analysis of agrochemical residues [8].

Enzyme-linked immunosorbent assay (ELISA) tests for laboratory analysis of MPA in microwells have been developed [9]. These laboratory assays were applied to the quantitation of MPA in animal tissues. For application to matrices of food importance, we have redeveloped the assay in new formats to make the assays more rapid and suitable for field use.

Up to now several immunoassays for MPA analysis have been reported which all use enzyme as tracer [10, 11]. Time-resolved fluoroimmunoassays (TR-FIAs) have been utilized in the analysis of veterinary drugs as well [12, 13]. In general, TR-FIA can offer better sensitivity than the more traditional ELISAs due to the unique fluorescent properties of the lanthanide chelates and the time-resolved measurement mode, which enable the specific fluorescence to be measured after the background fluorescence has already declined [14].

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In this paper, we describe the development and validation of a novel, rapid, trace method for the screening of MPA residues in pork tissues by using TR-FIA. This method was compared with ELISA and validated by applying to the analysis of field pork tissues samples from the local food markets.

Materials and methods

Reagents

Polycolonal rabbit antibody against medroxyprogesterone acetate with no significant cross reactivities with other chemicals was from our laboratory [9]. medroxyprogesterone acetate, apo-transferrin(human) and d-sorbitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit IgG, bovine serum albumin (BSA), acetonitrile and methanol were obtained from Boao Biotech Co., Ltd. (Shanghai, China). Dry *N*,*N*-dimethylformamide from Fluka (Buchs, Switzerland). Glycine and diethylenetriaminepentaacetic acid (DTPA) were purchased from Merck (Darmstadt, Germany). Germall II and intrinsically fluorescent europium chelate [2,2′,2″,2‴({4 – [(4 – isothiocyanatophenyl) ethynyl] pyridine -2,6-diyl}bis-(methylenenitrilo)) tetrakis(acetato)]-europium(III) was from Chinese Academy of Inspection and Quarantine (Beijing, China) [15]. DELFIA Assay Buffer, Wash Solution, Enhancement Solution and Victor 1420 Multilabel Counter were obtained from PerkinElmer Life and Analytical Sciences (Shanghaai, China). Non-coated, yellow (UV-quenched, low-fluorescence) MaxiSorp microtitration wells were purchased from Nunc A/S (Roskilde, Denmark).

Preparation of anti-rabbit IgG coated microtitration wells

Goat anti-rabbit IgG was activated with a 6-fold volume excess of 100 mM glycine-HCl, pH 1.9. The reaction was let to proceed for 15 min with slow shaking, after which the IgG was immediately added to 50 mM phosphate buffer, pH 7.0, to a final concentration of 8.3 μg mL^{-1}. The microtitration wells were coated with the activated IgG (120 μL per well) and incubated overnight at room temperature in a humid environment. The wells were washed twice with DELFIA Wash Solution and then saturated with 50 mM NaH_2PO_4, pH 4.5, containing 6% sorbitol, 0.1% BSA and 0.05% Germall II (250 μL per well). After an overnight incubation at room temperature in a humid environment, the wells were aspirated to dryness and stored in sealed packages with desiccant at 4°C.

Preparation of labelled analyte

MPA was activated as described by our laboratory [9]. The activated MPA was conjugated to 1 mg of apo-transferrin, dissolved in 500 μL of 50 mM phosphate buffer, pH 7.2, by using a 100-fold molar excess of MPA. The reaction was carried out overnight at room temperature with slow shaking. The MPA-apo-transferrin conjugate was labelled overnight at room temperature with a 60-fold molar excess of europium chelate in a volume of 1 mL in 50 mM carbonate buffer, pH 9.2. The conjugate was purified by gel filtration on a Superdex 200 HR 10/30 column (Boao Biotech Co., Ltd. Shanghai, China) with 50 mM Tris-HCl, pH 7.2, containing 0.9% NaCl, 0.05% NaN_3, as elution buffer at a flow rate of 20 mL h-1 and 0.5 mL fractions were collected. The fractions containing the labelled analyte were tested and those fractions having a good inhibition profile and high signal level were pooled to form the label stock. all reagents of the detection were stored at 4–8°C. The guaranteed shelf life of the reagents was six months.

Sample preparation

The stock solution of MPA (1 mg mL^{-1}) was prepared in methanol. The stock solution was stable at least 3 months when stored at 4°C. The working solutions were prepared daily in de-ionised water.

Swine pork tissues were collected from the lab farm of our university and known to have been reared under MPA-free conditions. These tissues were stored at −40°C until required.

Into a glass universal (25 mL) finely chopped pork tissues (2.5 g) was weighed and acetonitrile (7.5 mL) added, and spiked with appropriate concentrations of MPA when necessary. The samples were homogenised for 30 s and 1 M HCl (2.5 mL) added. Petroleum ether (5 mL) was added and the samples mixed using an end-over-end mixed for 15 min, followed by centrifugation at 3450 g for 10 min. The ether layer was discarded and the liquid phase was decanted into a glass test-tube and reduced to approximately 5 mL under a gentle stream of nitrogen on a Driblock and sample concentrator at 100°C. The reduction took approximately 20 min. The supernatant was transferred to a clean test-tube and NaOH (5 M, 3 mL) added. Dichloromethane (10 mL) was added and the samples mixed using an end-over-end mixer for 15 min, followed by centrifugation at 3450 g for 10 min. The extracts were passed through phase separation columns (6 mL) and the dichloromethane layer was collected in clean test tubes. The dichloromethane layer was evaporated to dryness (approx. 10 min) under nitrogen, using a Driblock and sample concentrator at 80°C, resuspended in ethanol (250 μL) and

vortexed for 15 s. Finally, DELFIA dilution buffer (2250 μL) was added.

Time-resolved fluoroimmunoassay
for medroxyprogesterone acetate

The medroxyprogesterone acetate antibody was diluted to a suitable working titer with DELFIA Assay Buffer and attached to the anti-rabbit IgG coated microtitration wells in a volume of 50 μL per well. After a 1-h incubation, the wells were washed four times with DELFIA Wash Solution. The extracted samples (25 μL per well) and labelled analyte (25 μL per well), which was diluted to an appropriate concentration with 100 mM phosphate buffer, pH 7.2, containing 0.4%BSA, 0.05%NaN_3 and 20 μM DTPA, were added to the wells and incubated for 30 min. The wells were washed six times, after which 200 μL of DELFIA Enhancement Solution was dispensed to each well. After a 15-min incubation period, the fluorescence signal was measured with a Victor 1420 Multilabel Counter using the default settings for europium measurement. The B/B_0 ratio was calculated by dividing the fluorescence signal of a sample/standard by the fluorescence signal of a zero sample/standard.

Reliability

The medroxyprogesterone acetate immunoassay was validated according to Commission Decision 2002/657/EC [16]. The limits of detection (LOD) of the assay was defined as the concentration corresponding to the mean signal of 20 blank samples plus 3 × standard deviation. The limits of quantification (LOQ) for a qualitative screening method was established by analyzing 20 spiked samples at a selected concentration level exceeding the concentration of LOD. The intra-assay variation was determined at six concentration levels ($n = 6$). The interassay variation (repeatability) was determined with six samples at each of the six concentrations over 3 days ($n = 18$). The recovery was assessed in a similar manner, except that the samples were allowed to stand overnight prior to analysis ($n = 6$).

Determination of field samples by LC-MS-MS

An Agilent 1100 series LC system (Delaware, USA) including G1313 A 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, USA) 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^{-1} was used. Injection volume of 10 μL was used for all analyses (Fluid Management System Inc., Minneapolis, USA). The gradient was as follows: for 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 ESI source (HP Lab, Palo Alto, CA, USA). 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^{-1} and the TurboIonSpray probe temperature was maintained at 550°C. The nebulizer and curtain gases were, respectively, 12 and 8 L min^{-1}, while the gas pressure in the collision cell was set at 3.4×10^{-5} Torr. Multiple reaction monitoring (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. Ion pair for quantification of MPA was 345.2/123.1

Table 1 Cross reactivity of MPA antisera with anabolic steroids

Anabolic steroid analog	Cross reactivity(%)
Compound	TR-FIA
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	<0.1
17-propionate	<0.1
Deoxycorticosterone	<0.1
17α-Hydroxyprodesterone	<0.1
Prednisolone	<0.1
Cortisol	<0.1

Results and discussion

Cross-reactivity

The specificity of the polycolonal antibody was evaluated using similar compounds structurally related to MPA and the results are presented in Table 1. The polycolonal antibody exhibited high cross-reactivity with MPA. Therefore, the polycolonal antibody was specific to MPA and can be used in immunoassays for screening MPA in animal tissues.

Reliability

If the assay was used in a qualitative manner, only positive and negative control samples were extracted with the

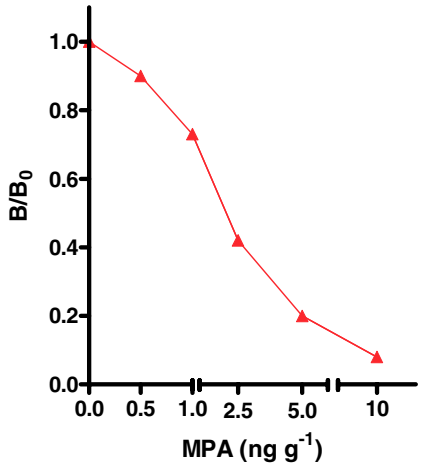

Fig. 1 Standard curve for medroxyprogesterone acetate residues in pork tissues ($n = 6$)

unknown samples. These controls ensured the functionality of the assay (negative control) and established the critical signal level (positive control), to which the unknown samples were compared in order to classify them as compliants or non-compliants. For quantitative results, a standard curve using extracted samples was required. A typical standard curve for medroxyprogesterone acetate residues in pork tissues is shown in Fig. 1. The intra- and interassay variations were determined at six selected concentrations. The results were shown on Table 2 and Table 3. Intra-assay variation was less than 10% with six replicate wells at each of the selected concentration levels. The interassay variation was assessed similarly by analyzing six replicate samples on three separate occasions and was from 9.7 to 12.7%. The recoveries, which were determined with spiked samples, were from 87.3 to 108.3%, and the coefficient of variation was from 6.3 to 12.1%.

The limit of detection (LOD), defined as mean $+ 3 \times$ SD for MPA was determined by repeated analysis ($n = 20$) of MPA-free pork tissues samples [17]. The limit of quantification (LOQ) was defined as the MPA concentration of samples giving a B/B_0 ratio of approximately 0.8 with a coefficient of variation (CV) of less than 20%.

Table 2 Validation data for medroxyprogesterone acetate immunoassay

Concentration (ng g^{-1})	Coefficient of variation (%)	
	Intra-assay ($n = 6$)	Interassay ($n = 18$)
0.1	8.6	12.7
0.5	7.9	12.1
1	6.8	10.8
2	5.7	9.7
4	6.4	11.3
5	7.6	10.8

Table 3 Recovery for medroxyprogesterone acetate residue analysis determined at six concentration levels ($n = 6$)

Concentration (ng g^{-1})	Mean recovery (%)	Coefficient of variation (%)
0.1	108.3	12.1
0.5	106.2	11.6
1	101.2	9.3
2	87.3	6.3
4	88.4	10.1
5	87.7	8.7

The LOD was established as 0.06 ng g^{-1} by analyzing 20 blank samples. The LOQ was determined with 20 spiked samples and the LOQ was 0.8 ng g^{-1}.

The performance of the new TR-FIA method was compared with that of ELISA kit, which was developed by our laboratory, using MPA-spiked samples ($n = 25$, spiked concentration of MPA was more than 0.1 ng g^{-1}). MPA concentrations measured by the TR-FIA and the ELISA were comparable (Fig. 2). Linear regression analysis showed good correlation, with r^2 values 0.97.

Determination of field samples by LC-MS-MS

To further demonstrate reliability of the TR-FIA for the determination of MPA residues in animal tissues, 30 pork tissues samples from local food markets were analyzed using the TR-FIA and LC-MS-MS methods. MPA was found in 5 out of 30 pork tissues samples by both methods. MPA concentrations in these samples are presented in Table 4. There was no false-negative or false-positive result by the established TR-FIA, compared to the LC-MS-MS. The TR-FIA results were consistently higher than the LC-MS-MS results; this difference was probably resulted from that immunoassay

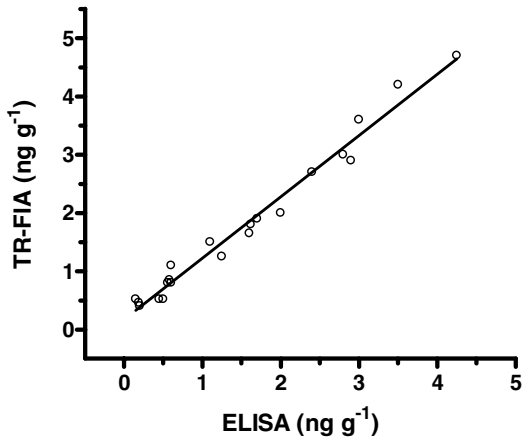

Fig. 2 Correlation between MPA concentrations measured by TR-FIA and by an ELISA kit in pork tissues samples

Table 4 MPA residues in pork tissues samples purchased from local food markets ($n = 3$)

Samples	MPA (ng g^{-1}, mean ± SD)	
	TR-FIA	LC-MS-MS
1	0.7 ± 0.1	0.5 ± 0.2
2	4.6 ± 0.3	2.1 ± 0.4
3	5.8 ± 0.6	4.3 ± 0.3
4	1.9 ± 0.3	1.5 ± 0.4
5	6.3 ± 0.7	4.9 ± 0.6

often overestimates the analyte concentration due to interference from other steroids and lipids.

Conclusions

The current study has resulted in the development and validation of a competitive TR-FIA for medroxyprogesterone acetate residues in pork tissues. The proposed technique has many advantages over current published methods. This rapid screening method could be applied to routine residue analysis. But the cumbersome preparation procedure of this method will still be developed in the future.

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