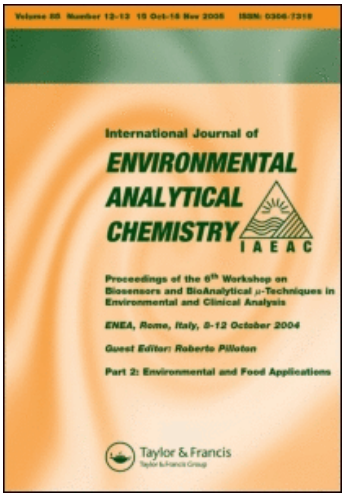

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Development of colloidal gold-based immumochromatographic assay for the rapid detection of medroxyprogesterone acetate residues in biological materials

Chi-Fang Peng[a]; Li-Qiang Liu[a]; Chuan-Lai Xu[a]
[a] School of Food Science and Technology, Southern Yangtze University, JiangSu Province, China

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Development of colloidal gold-based immumochromatographic assay for the rapid detection of medroxyprogesterone acetate residues in biological materials

CHI-FANG PENG, LI-QIANG LIU and CHUAN-LAI XU*

School of Food Science and Technology, Southern Yangtze University, 214036 WuXi, JiangSu Province, China

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A competitive colloidal gold-based immunoassay in lateral-flow format for the rapid detection of medroxyprogesterone acetate (MPA) in biological materials was developed. A nitro-cellulose membrane strip was separately coated with goat anti-rabbit IgG (control line) and MPA hapten-OVA conjugate (test line). Anti-MPA polyclonal antibody labelled with colloidal gold particles was first incubated with MPA. The limit of detection for lateral flow was $5\,ng\,g^{-1}$ for detecting an MPA standard solution, and the limit of detection was $10\,ng\,mL^{-1}$ for detecting the MPA spiked in pig urine and $10\,ng\,g^{-1}$ for spiked in pig liver. The results were confirmed by high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and indicated that there was a good agreement between both methods ($R^2 = 0.976$). The assay time for the test was less than 5 min, suitable for rapid testing on site.

Keywords: MPA; Lateral-flow; Colloidal gold-based immunoassay

1. 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 tumours. Among veterinary drugs, MPA is considered a hormone which can accelerate fattening [1]. The presence of traces of MPA in animal tissues poses a potential hazard for consumers. According to the European Commission Decision 2002/657/EC [2], the maximum residue level (MRL) in animal tissues is less than $0.1\,ng\,g^{-1}$. 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 [3–8]. However, these methods require well-equipped laboratories, trained personnel, and 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.

*Corresponding author. Fax: +86-510-85881769. Email: XCL@sytu.edu.cn

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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 on-site testing by technically competent personnel. Immunoassay is the most suitable testing method for rapid field analysis of agrochemical residues [9].

Enzyme-linked immunosorbent assay (ELISA) tests for laboratory analysis of MPA in microwells have been developed [10]. 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.

Until now, several immunoassays for MPA analysis have been reported which all use enzyme as tracer [11, 12]. In this article, we describe the development of one-step colloidal gold-based lateral-flow assays for the detection of MPA.

2. Experimental

2.1 *Chemicals and reagents*

Nitro-cellulose hi-flow plus membrane were purchased from Millipore (catalogue no. INYC00010, Bedford, MA). Semi-rigid polyethylene sheets and adhesive tape were purchased from a local market. Filter paper, goat anti-rabbit IgG, and analytical-grade buffer chemicals were purchased from Boao Biotech Co., Ltd (Shanghai, China).

Bovine serum albumin (BSA) and ovalbumin (OVA) were from Sino-American Biotechnology Company (imported in bulk); phosphate-buffered saline (PBS) was from Shanghai Chemical Reagent Station (AR) (Shanghai, China). Chloroauric acid and other chemicals were purchased from Sigma (St. Louis, MO). To avoid contamination, all glassware was baked for 4 h at 400°C prior to use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination.

2.2 *Apparatus*

The following equipment was used: Camag Linomat 5 automatic TLC (thin-layer chromatography) sampler (CAMAG, Muttenz, Switzerland) and an FTS EI585-Q freeze dryer (American Stone Ridge). Ultra-pure water was produced using the Milli-Q Ultra-pure System. Amino-propyl solid-phase extraction cartridges containing 500 mg materials (3 mL) was purchased from Waters Co. (Milford, MA).

An Agilent 1100 series LC system (Delaware) including a G1313A quaternary pump, a G1313A autosampler, and a 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) with a particle size of 5 μm. A binary gradient consisting of acetonitrile (A) and purified water (B) at a flow rate of 0.3 mL min^{-1} was used. An injection volume of 10 μL was used for all analyses (Fluid Management System Inc., Minneapolis, MN). The gradient was as follows: for androgens and progestogens, the linear gradient protocol was 50% A to 100% A within 15 min. Mass spectrometry was performed using an API3000 tandem triple-quadrupole mass spectrometer equipped with a TurboIonSpray ESI source (HP Lab, Palo Alto, CA). The ion-spray voltage

was 3000 V. High-purity nitrogen was used as nebulizer, heater, curtain, and collision gas. The heater gas was set at 7.5 L min^{-1}, and the TurboIonSpray probe temperature was maintained at 550°C. The nebulizer and curtain gas flow rates were 12 and 8 L min^{-1}, respectively, 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 a unit resolution, and the dwell time was 150 ms.

The conditions of total ion chromatography of standard progestogens in LC-MS-MS analysis were as follows: TEM: 550°C; IS: 3000 V; CAD: 7.0; mobile phase: acetonitrile (A) and water (B), gradient used for elution: 0.01 min A 50% (φ), 15 min A 100% (φ), 10 μL injection volume (1 μg mL^{-1}). The conditions of LC/MS/MS chromatography of progestogens in spiked samples were as follows: TEM: 550°C; IS: 3000 V; CAD: 7.0; mobile phase: acetonitrile (A) and water (B), linear gradient used for elution: 0.01 min A 50%, 15.0 min A 100%, 10 μL injection volume [10].

2.3 *Preparation of immunoassay reagents*

The synthesis of MPA haptens used to develop the immunoassay has been described [10]. Haptens were then coupled to the following carrier proteins: BSA for use as immunogens, and OVA for coating on the membrane. The protocols for immunization of rabbit and purification of polyclonal antibody against MPA were the same as that described in a previous study [10].

2.4 *Preparation of colloidal gold*

Nanometre colloidal gold was prepared according to a previously published procedure [13]. The procedures for preparing gold colloidal suspension were as follows: 100 mL of 0.01% $HAuCl_4$ solution (in deionized water) was boiled thoroughly, and then 120 mL of a solution of 1% trisodium citrate was added under constant stirring. After the colour of the solution had changed from blue to dark red within 2 min, the solution was boiled for another 5 min. The strength of the colour is closely related to the size and quality of colloidal gold particles. The size of gold colloidal particles was directly dependent on the amount of trisodium citrate used in its preparation process. The optimal size selection of colloidal gold has been described in the literature [14–16]. The obtained gold colloidal suspension could be stored at 4°C for several months and supplemented with 0.05% (m v^{-1}) of sodium azide. With the scan between 500 and 600 nm, there is only one maximum absorbent wavelength: 525 nm. The particles thus obtained were checked using a transmission electron microscope, and the average diameter of these uniformly sized particles was found to be 40 nm.

2.5 *Labelling antibody with colloidal gold*

Gold markers have the characteristics of binding proteins non-covalently without changing their bioactivity. This adsorption leads to the formation of a 'protein–gold complex' [17, 18]. The pH of colloidal gold solution for anti-MPA polyclonal antibody conjugation was adjusted to pH 9.0 with 0.1 M K_2CO_3. Before conjugation, the optimal

concentration of antibody for conjugation was determined. With gentle stirring, 2.4 mL of purified anti-MPA antibody (0.4 mg mL^{-1}) was added drop by drop to 100 mL of a pH-adjusted colloidal gold solution. After overnight incubation at 4°C, the mixture was centrifuged at 6000 *g* and 4°C for 30 min, and the pellet was resuspended in 10 mL of conjugate storage buffer (2 mM sodium borate containing 0.1% BSA and 0.1% sodium azide, pH = 7.2) and diluted for use.

2.6 *Colloidal gold-based lateral-flow immunoassay*

2.6.1 Preparation of membrane strip for lateral flow. The nitro-cellulose membrane, which carried the positive electric charge, was cut into sections (2.5 × 0.5 cm). The test line was coated with MPA hapten conjugate, which was applied to each membrane in a volume of 1 µL containing 1 µg of MPA hapten conjugate with a Camag Linomat 5 automatic TLC sampler. The control line was coated with 0.5 µL of goat anti-rabbit IgG diluted 1 : 50 in PBS buffer (pH 7.2). The distance between the test line and control line was 2 mm. The test strips were dried at 37°C for 30 min. The remaining protein-binding sites of the membrane were blocked by immersing the strips in PBS containing 1% BSA at 37°C for 30 min. The test strips were washed and dried, and the coated test strips were stored in a desiccator at 4°C.

2.6.2 Procedure of lateral-flow immunogold assay. The test strip was pasted onto a plastic backing with adhesive. Dried filter paper acted as an absorbent pad (figure 1). Ninety microlitres of MPA standards in 5% methanol (prepared in PBS-0.05% Tween) were mixed with 30 µL of gold–antibody conjugate, after incubation for 5 min, and a 50 µL mixture of gold–antibody and sample containing MPA were pipetted into the bottom of the strip (sample application site). After the liquid reagent migrated towards the test line, a different colour intensity on the test line could be observed with the

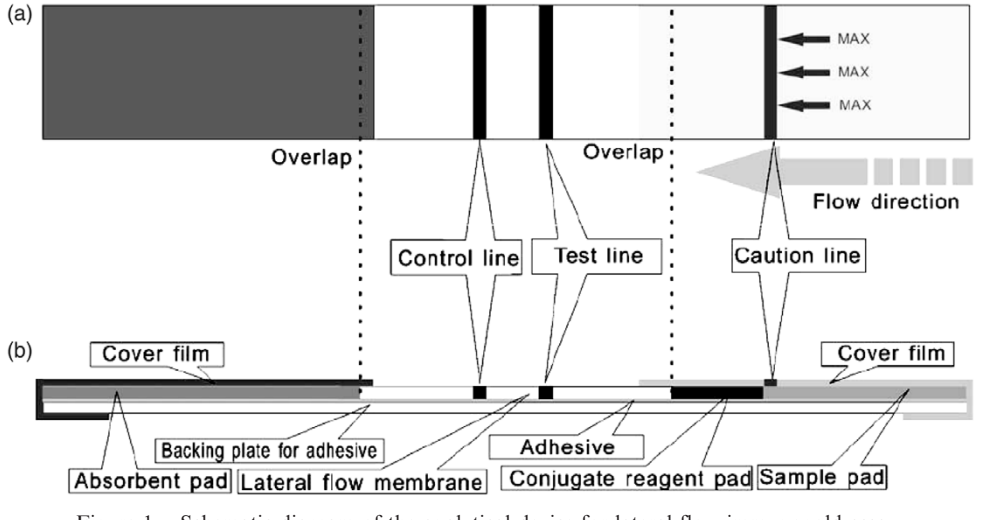

Figure 1. Schematic diagram of the analytical device for lateral-flow immunogold assay.

naked eye. The colour of the test line was compared with the test line of a negative control strip (without MPA).

2.7 *Rapid extraction and HPLC/MS/MS analysis*

2.7.1 Urine and liver samples. Urine and liver samples from pig with a known history and certified as free of MPA were used in the studies from our university lab farm. Urine samples were filtered through a Whatman no. 1 filter paper and stored frozen (−20°C) in aliquots of 10 mL until analysed. Liver samples were homogenized, accurately weighed (10 g wet mass), and stored at −20°C until used.

2.7.2 Extraction of liver. A 10 g portion of minced liver was homogenized with 30 mL of an ethyl acetate–isopropanol (7 : 3 v/v) mixture. The tube containing the homogenate was centrifuged at 5000 *g* for 20 min at 5°C. The supernatant was evaporated under vacuum, and the dry residue was dissolved in 10 mL of 0.01 M perchloric acid. The resulting mixture was transferred to another tube and centrifuged at 5000 *g* for 10 min at 5°C. The supernatant was transferred to a clean tube, the pH adjusted to 8.5 with 2 M NaOH, and the supernatant recentrifuged if a precipitate was formed. The whole solution was cleaned up by solid-phase extraction (SPE).

2.7.3 Extraction of urine. A 10 mL portion of urine sample was diluted with 50 mL of 0.05 M borate buffer, pH 8.5, and centrifuged at 5000 *g* for 10 min at room temperature. The whole was used for clean-up by SPE.

2.7.4 SPE clean-up. The SPE cartridges were washed with 5 mL of methanol, 5 mL of water, and 5 mL of 0.05 M borate buffer, pH 8.5. The previously prepared test solutions were applied to the preconditioned columns and passed through in about 10 min. Then, they were washed successively with 2 mL of water, 1 mL of 0.1 M acetate buffer, pH 4.0, and methanol. After drying under vacuum for 5 min, the analyte was eluted with dichloromethane–isopropanol–concentrated ammonia (80 : 20 : 2 v/v/v). The solutions were evaporated under nitrogen, and the dry residues were dissolved in 200 μL of phosphate buffer, pH 7.4, for analysis. The HPLC/MS/MS analytical method was the same as that described in a previous article [10].

3. Results and discussion

3.1 *Assay of cross-reactivity*

The cross-reactivity (CR) of MPA antisera with anabolic steroid analogues was carried out by ic-ELISA. The CR of medroxyprogesterone is 96%; the CR of epitestosterone, nandrolone 17α-methyltestosterone and testosterone-17-propionate is less than 0.1%; and the CR of 17β-oestradiol, oestriol, 17α-ethynyloestradiol, oestrone, 17β-oestradiol-3-benzoate, and progesterone (PG) is less than 20%. The results are presented in table 1.

Table 1. Cross-reactivity of MPA antisera with anabolic steroids analogues.

Anabolic steroid analogue	Cross-reactivity (%)
Medroxyprogesterone	96
Oestradiol	21
17β-Oestradiol-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

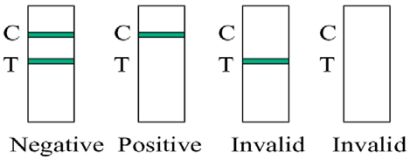

Figure 2. Illustrations of immunochromatographic test results.

3.2 *Determination of test results*

The assay is designed such that if there is no MPA present in the test, the gold–antibody will accumulate on the nitrocellulose membrane where they are trapped by the immobilized MPA–hapten conjugate while liquid flows through the test strip. For samples containing MPA residues, the binding sites on the specific antibody molecules will be occupied first by MPA, leaving fewer binding sites for MPA hapten–OVA conjugate on the membrane. Consequently, less colloidal gold-labelled antibody will remain at the hapten–OVA location on the nitrocellulose membrane. Thus, the degree of intensity of the gold colour on the test line is the inverse of MPA concentration in the sample, and the visual result is immediately observable.

The test result was judged visually. The goat anti-rabbit IgG and MPA hapten–OVA conjugate were separately spotted onto the control line (C) and test line (T) shown in figure 2. If there was no colour present in the control line, the test could be considered invalid. First, the antibody–gold conjugate could react with MPA in the sample, and then the remaining antibody–gold conjugate would bind with the hapten–OVA conjugate located on the test line on the membrane. The assay developed is a competitive assay so the colour intensity of the test line is inversely correlated with the concentration of MPA in the original sample. Test lines were also scanned with a BioDot TSR3000 Membrane Strip Reader (BioDot, CA).

3.3 *Detection limit of MPA test strip*

The main purpose of the assay was to allow visual evaluation, so it was only used as a qualitative assay to detect contamination at a threshold level. For this, the colour

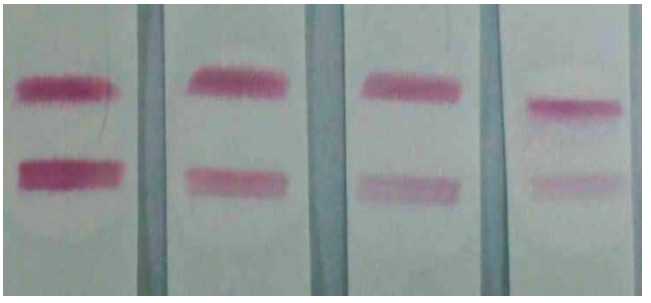

Figure 3. Lateral-flow immunogold assay. Top line: control line (goat anti-rabbit IgG); bottom line: test line (MPA hapten–OVA). MPA concentration from left to right: 0, 5, 10, 20 ng g^{-1}. The color intensity of 5 ng g^{-1} was clearly distinguishable from that of the negative control.

intensity of the test line must be high enough to be seen and enable differences in colour intensity to be observed between negative control and samples. For this purpose, various parameter settings were tested to determine (1) the optimal immobilization concentration of MPA hapten–OVA conjugate (l.0, 2.5, 5.0 μg), (2) the optimal ratio of gold–antibody conjugate and MPA (1 : 1, 1 : 2, 1 : 3), and (3) the optimal incubation time (1, 5, 10 min). The optimal immunoreagent concentration was selected as a clear colour appearing in the negative control with the shortest time, and comparison of the intensity of colour among samples and control could be easily distinguished visually.

The optimal conditions for lateral-flow colloidal gold immunoassay were as follows: MPA hapten–OVA coated on the membrane at 1 μg $strip^{-1}$; gold–antibody conjugate and MPA in the ratio of 1 : 3 and incubation time of 5 min. As shown in figure 3, 5 ng g^{-1} of MPA caused a slight but distinguishable difference compared with the negative control for lateral-flow immunoreagent colloidal gold assay. Thus, 5 ng g^{-1} of MPA was considered to be the detection limit for the lateral-flow assay.

3.4 *Reliability*

The reliability of the lateral-flow assay was determined by carrying out the test with uncontaminated samples spiked with MPA at concentrations of 1, 5, 10, 20, and 50 ng g^{-1} and analysed by the lateral-flow gold-based immunoassay. The results are shown in table 1. The recovery of 1 and 5 ng g^{-1} MPA spiked to the samples exceeded 120%, and CV values were more than 12%. Between the 10 and 50 ng g^{-1} level, the recovery of MPA was found to be less than 100% and CV less than 10%. Results indicate that the limit of detection was 10 ng mL^{-1} for detecting the MPA spiked in pig urine and 10 ng g^{-1} for MPA spiked in pig liver. Uncontaminated samples spiked with MPA concentrations at 10, 20, 30, 40, and 50 ng g^{-1} were also tested by lateral-flow gold-based immunoassay and confirmed by HPLC/MS/MS. The results are displayed in tables 2 and 3, and indicate that there is a good agreement between the data obtained with both methods. The linear regression equation between HPLC/MS/MS and IC-test data was $y = 1.18x + 0.547$ with a square of correlation coefficient of 0.976 and a slope of 1.18. This demonstrated a good correlation between the two methods.

A field analysis of 32 pork liver samples using the rapid lateral-flow colloidal gold-based immunoassay resulted in all 32 samples being judged negative.

Table 2. Analytical recovery of MPA spiked to urine and liver samples.

Spiked level ($ng\,g^{-1}$)	Recovery ± SD (%) Urine	CV (%)	Recovery ± SD (%) Liver	CV (%)
1	121.36 ± 2.43	13.48	144.23 ± 7.14	13.78
5	139.41 ± 1.44	14.67	141.11 ± 4.31	12.49
10	97.31 ± 2.04	9.45	98.11 ± 1.91	8.38
20	89.43 ± 3.01	9.86	88.36 ± 3.42	9.01
50	91.31 ± 2.86	8.23	87.41 ± 2.36	8.19

CV: coefficient of variation; SD: standard deviation.

Table 3. Comparison of results obtained by gold-based immunoassay and HPLC/MS/MS ($n = 6$).

Samples	Spiked level	Test strips Mean ± SD	Results of HPLC Mean ± SD
Urine ($ng\,mL^{-1}$)	10	7.31 ± 0.38	8.72 ± 0.42
	20	16.38 ± 0.87	17.13 ± 0.78
	30	28.31 ± 1.08	29.41 ± 1.21
	40	37.69 ± 1.21	38.31 ± 1.48
	50	48.37 ± 1.49	46.43 ± 1.07
Liver ($ng\,g^{-1}$)	10	6.89 ± 0.49	6.01 ± 0.57
	20	16.77 ± 1.34	16.45 ± 1.21
	30	26.27 ± 1.23	25.67 ± 1.69
	40	35.78 ± 1.77	36.18 ± 1.34
	50	46.33 ± 2.36	45.34 ± 1.28

The confirmation step with the HPLC/MS/MS analysis also resulted in negative samples. This means that neither false negative nor false positive results were obtained in the survey. The assay process was very simple and easy to use. The whole analysis of colloidal gold-based immunoassay was completed in less than 5 min, whereas HPLC/MS/MS takes several hours.

4. Conclusions

The major advantages of the one step strip test were that results could be obtained within 5 min and that all reagents needed were included in the strip. The strips could be used to detect the MPA residue in pig urine and liver in spots. The interpretation of the results was visual, and it was found that these assays could be used as convenient qualitative tools for the rapid screening of MPA residues in animal tissue samples. The method provides only a preliminary, semi-quantitative result. The results could be used to judge whether the MPA concentration remaining in the sample was higher than the detection limit or not.

The method producing the one step strip for MPA could be used for reference in the development of one step strip for the detection of other anabolic steroid residues.

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