Preparation of an anti-dexamethasone monoclonal antibody and its use in development of a colloidal gold immunoassay

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\textbf{ABSTRACT}
A lateral flow colloidal gold (CG) immunoassay strip has been developed for detection of dexamethasone (DEX) residues in milk samples. For this purpose, an anti-DEX monoclonal antibody (McAb), based on a DEX succinic anhydride derivative hapten, was prepared and characterized. The McAb showed a high specificity to DEX, the half inhibitory concentration of the antibody was 0.095 ng/mL, its limit of detection (LOD) was 0.017 ng/mL, and its linear range of detection was 0.034–0.265 ng/mL. The developed CG immunoassay had a visual cut-off value of 0.3 ng/mL in phosphate buffered saline (PBS) and 0.5 ng/mL in milk samples. Each test requires 10 min. Analysis of DEX in milk indicated that the results of strip assay had a strong agreement with indirect competitive enzyme-linked immunosorbent assay. Therefore, the CG immunoassay is a sensitive screening method for semi-quantitative and qualitative detection of DEX residues in milk samples.

\textbf{ARTICLE HISTORY}
Received 11 April 2017
Accepted 13 April 2017

\textbf{KEYWORD}
Dexamethasone; antibody; colloidal gold strip; milk

\section*{Introduction}

Dexamethasone (DEX) is a synthetic glucocorticoid which is frequently used in veterinary practice for treating some diseases of farm animals (Bailey et al., 1973; Tatone et al., 2016). DEX has anti-inflammatory and anti-allergic effects in the primarily therapeutic use of animal (Chu et al., 2014; Sami, Mohri, Seifi, & Chavatte-Palmer, 2015). And, DEX also promotes water retention in meat, lipid metabolism, and beneficial effects on other growth promoters (Yuan et al., 2008). However, their strong pharmacological activity may be making an accumulation of these molecules in animal bodies (Cannizzo et al., 2011; Ferranti et al., 2013; Hansen, Laborde, Wall, Holson, & Young, 1999). A study by Van den Hauwe, Schneider, Sahin, Van Peteghem, and Naegeli (2003) found that the conventional therapeutic use of DEX may cause its concentration in animal tissues exceeding the maximum residue limits (MRLs) more than 10 times. Thus, for ensuring the health of human, the MRLs for DEX equal to 2 μg/kg in liver, 0.75 μg/kg in muscle and kidney, and 0.3 μg/kg in milk have been set by the European Commission (Commission, 2009).
Recently, various analytical methods have been developed for detecting DEX, such as high-performance liquid chromatography (Bhargava et al., 2016; Dési, Kovács, Palotai, & Kende, 2008; Lasić, Bobarević, & Nikolin, 1989; Tsuei, Ashley, Moore, & McBride, 1978), gas chromatography–mass spectrometry (Amendola, Garribba, & Botrè, 2003; Bagnati et al., 1996; Huetos Hidalgo, Jiménez López, Ajenjo Carazo, San Andrés Larrea, & Reuvers, 2003), liquid chromatography–mass spectrometry (LC-MS) (Chen et al., 2011; Creaser, Feely, Houghton, Seymour, & Teale, 1996), radio-immunoassay methods (Meikle, Lagerquist, & Tyler, 1973), and enzyme immunoassay (Hassan, Rowell, Hambleton, & Jackson, 1998; Vdovenko, Gribas, Vylegzhanina, & Sakharov, 2012; Yadav et al., 2013; Yoshino, Yoshiharu, Noriko, Kiyoshi, & Fukuko, 1992). Each method has its own strong and weak points. To reduce the risk of people exposure to DEX, a suitable high-throughput analytical technique would be developed to screen a range of food and feed samples. The instrumental methods require expensive instrumentation, extensive sample cleanup, professional specialist, and long time. The immunoassays such as enzyme-linked immunosorbent assay (ELISA) also require time and personnel. So, these methods are not suitable for screening a large amount of samples at the least time. Compared with the above methods, the immunochromatographic strip assay has some advantages on the routine screening, including easy to carry out, high throughput, quick results (within 5–10 min), and so on. In this study, we attempt to establish a semi-quantitative or qualitative colloidal gold (CG) immunoassay for fast detection of DEX residues in milk.

**Materials and methods**

**Chemicals and materials**

DEX, betamethasone (BET), hydrocortisone (HDS), fludrocortisone (FDS), estradiol (E2), progesterone (P) and testosterone (T) were purchased from J&K Scientific Ltd (Beijing, China). Bovine Serum Albumin (BSA), ovalbumin (OVA), succinic anhydride (HS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Freund’s complete adjuvant (FCA), Freund’s incomplete adjuvant (FIA), 3,3′,5,5′-tetramethylbenzidine, and gold chloride trihydrate were obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI-1640 cell culture medium, hypoxanthine–aminopterin–thymidine supplement, hypoxanthine–thymidine supplement, polyethylene glycol (PEG) 1500 and fetal calf serum were purchased from Gibco BRL (Paisley, UK). All the reagents and solvents were of analytical grade or higher.

The hapten and antigen were characterized by UV/VIS scanner (Bokin instruments, Tsushima, Japan); all buffer solutions were prepared with ultrapure water produced by Waters Maldi Synapt Q-Tof MS (Waters, Shanghai, China); the results of indirect competitive (ic) ELISA was measured by Multiskan MKS microplate reader (Thermo Lab-systems Company, Beijing, China); and other instruments used in this study are as follows: membrane dispenser (Xinqidian Gene-Technology Co. Ltd, Beijing, China), vortex machine (Shanghai Huxi Analysis Instrument Factory Co., Ltd, Shanghai, China), and water bath (Shanghai Instrument Group Co., Ltd, Supply & Sales Co., Shanghai, China).
**Preparation of the hapten and antigen**

A scheme for the production of DEX–HS conjugates by the succinic anhydride method (Zhang et al., 2016) is shown in Figure 1. The steps are briefly described as follows.

DEX (100 mg) and succinic anhydride (28 mg) were dissolved in 8 ml of anhydrous pyridine and refluxed at 60°C for 12 h. Then, the reaction residue dried by rotary evaporation was dissolved in 6 ml of 50% methanol aqueous solution and extracted three times with ethyl acetate. After 10% HCl (v/v) was added to the organic layer, the white precipitate was collected and dried at 37°C in a drying oven. An LC-MS analysis confirmed that the production met our requirements.

The DEX–HS–BSA conjugate and the DEX–HS–OVA conjugate prepared with the active ester method (Gu, Liu, Song, Kuang, & Xu, 2016) were used as immunogen and coating antigen, respectively. 6.6 mg of DEX, 4.5 mg of NHS, and 7.8 mg of EDC were dissolved in 0.3 mL of dimethyl formamide and stirred for 6 h at room temperature. The mixture solution was divided into two parts at 0.2 and 0.1 mL, respectively; the first part was added dropwise into 10 mg of BSA dissolved in 3 mL of 0.1 M sodium carbonate–bicarbonate buffer (CB, pH 9.6) and the other was added 10 mg of OVA dissolved in CB. The solution was incubated overnight with continuous stirring. Then, the end products were dialyzed against 0.01 M PBS (pH 7.4) for 3 days and were stored at −20°C. The final antigens were characterized with UV/VIS spectroscopy.

**Production of monoclonal antibody against DEX**

Ten 6-week-old female BALB/c mice were subcutaneously injected (Hao et al., 2009; Xu, Xu, Ma, Kuang, & Xu, 2015), respectively, with antigen DEX–HS–BSA. The first immunizing dose consisted of 100 μg of antigen as an emulsion of PBS and FCA. Four sequential boosters were administered at 3-week intervals with 50 μg of immunogen emulsified in FIA. After each booster, the serum collected from the tail vessel of each mouse was detected for the antibody specificity by ic-ELISA. The mouse with the highest titer and the best specificity to DEX was chosen to be injected intraperitoneally with 25 μg of immunogen dissolved in 100 μL normal saline.

Hybridomas secreting anti-DEX antibodies were generated as described previously (Chen et al., 2016; Kong et al., 2015). Briefly, the splenocytes isolated from the target mice were fused with SP2/0 cell using PEG 1500 and then the fused cells were distributed into 96 well-culture plates. The supernatants from the plates were detected with an ic-ELISA after one week. Selected cells were subcloned by the limiting dilution method. Five female BALB/c mice (10 weeks old) were prepared to product ascites fluids, and then the monoclonal antibody (McAb) was purified with the caprylic-acid–ammonium-sulfate precipitation method (Liu, Hung, Lu, Chou, & Yu, 2014). After 3-day dialysis, the McAb was stored at −20°C.

![Figure 1](image) The scheme for the production of DEX–HS conjugate.
Characterization of McAb

The antibody was isotyped according to a mouse McAb isotyping kit. The sensitivity of the McAb was evaluated from its half inhibitory concentration (IC50) value, which is defined as the concentration of DEX needed to inhibit 50% of the maximum absorbance, the linear range to detect DEX defined as the concentration of DEX toward from 20% to 80% inhibition of the control, and the LOD value, which is equivalent to IC10.

The affinity constant ($K_{aff}$) was calculated by the method of Beatty (Beatty, Beatty, & Vlahos, 1987). The formula for calculating the same is as follows:

$$K_{aff} = \frac{(n - 1)}{2(n[Ab_t] - [Ab])},$$

where $[Ag]$ was concentration of coating antigen and $[Ab]$ was concentration of McAb at 50% of the ODmax, and $n = [Ag]/[Ag_t]$.

The specificity of the McAb was determined by evaluating the cross-reactivity (CR) of the analytes compared to DEX. The CR of the McAb against DEX, BET, HDS, FDS, estradiol (E2), P and testosterone (T) was calculated by the following equation:

$$CR(\%) = \frac{IC_{50}\text{of DEX}}{IC_{50}\text{of a related analogue}} \times 100\%.$$

Preparation of CG particles

The CG particles with an average diameter 17 nm were produced using previously reported methods (Song et al., 2016; Zhao et al., 2008). Briefly, 50 mL of 0.01% of chloroauric acid solution (HAuCl4) was heated to boiling point, and then 2.5 mL of 1% trisodium citrate solution was added with vigorous stirring. The color of the solution turned black within 20 s, and then quickly changed to wine-red. After the color change, the solution was boiled for another 10 min, then cooled to room temperature, and stored at 4°C for further use.

Labeling the McAb with CG

The anti-DEX McAb labeled CG particles was prepared across to the previous methods (Chen et al., 2009; Peng et al., 2009; Wang et al., 2016). Briefly, 8 µg antibody (0.1 mg/mL) diluted in PBS was added to 1 mL of CG solution (adjusted to pH 8.8 by 0.1 mol/L K2CO3) with mild shaking, and then the mixture was stirred for 45 min at room temperature. After blocking the free CG with 1 mL of 10% BSA aqueous solution, the solution incubated at room temperature for another 1 h. The labeled McAb washed by repeated centrifugation (20,000 g) at 4°C for 30 min with gold-labeled resuspension buffer (20 mmol/L Tris [pH 8.2], 0.1% Tween, 0.1% PEG, 5% trehalose, 5% sucrose, 5% Brij, 0.2% BSA). The precipitate was resuspended in the resuspension buffer and stored at 4°C for use.

Preparation of the immunochromatographic strip

The immunochromatographic strip consists of sample pad, nitrocellulose (NC) membrane, absorption pad, and polyvinylchloride (PVC) backing card. The coating antigen (1 mg/mL DEX–HS–BSA) and the goat anti-mouse IgG (1 mg/mL) were immobilized on NC membrane as the test and control lines (T/C) by dispenser,
respectively. The sample pad was saturated with 0.01 M PBS containing 1% BSA, 1% sucrose, and 0.2% Tween 20 and dried for 3 h at room temperature. The absorption pad was made from pure cotton linter filter paper. The sample pad, NC membrane containing T and C lines, and absorption pad were assembled on the PVC backing card sequentially.

**Test procedure and principle**

Fifty microliters of anti-DEX McAb labeled CG and 150 μL of standard DEX solution or sample extract were mixed and incubated for 5 min at room temperature. Then, the mixture was added to the sample pad and migrated toward the absorption pad. The results would be visually obtained after 5 min.

When DEX was absent from the sample, all of McAb labeled CG would be trapped by coating antigen immobilized on T line to form a red line. When DEX is present in the sample, it would compete with the immobilized antigen for limited amount of McAb labeled CG. Therefore, the color of T line depended on the amount of DEX in sample. The more DEX existed, the lighter the T line colored. When there was enough DEX to react with all of McAb labeled CG, T line would be invisible. Whether or not DEX was present in the sample, the C line would become red because of the reaction of McAb labeled CG and goat anti-mouse antibody. So the C line colored red would ensure that the test strip and procedure were correct.

**Sample pretreatment**

Milk samples purchased from local markets were confirmed to be DEX-free by LC-MS. In this study, milk samples required no processing before testing, and would be spiked by a series of DEX to validate the CG assay. Each sample was analyzed more than three times in this experiment.

**Results**

**Characterization of antigen**

The molecular weight of DEX is so low that it could not induce a specific immune response. So DEX must be conjugated with a carrier protein to generate the immunogenicity. In this work, DEX was derived by succinic anhydride method for generating the hapten. Then the hapten was, respectively, conjugated with BSA and OVA as immunogen and coating antigen. This selection is critical for producing a specific antibody to DEX.

As shown in Figure 2(a), BSA had an absorption peak at 280 nm, and DEX–HS had a peak at ~250 nm. After DEX–HS being conjugated with BSA, the absorption peak of DEX–HS–BSA exhibited the peak of BSA and shifted to the peak of DEX–HS. These phenomena were also present in the spectroscopy of DEX–HS–OVA (Figure 2(b)). That indicated that the hapten was successfully conjugated to the carrier protein.
Characterization of the McAb 6D7

The McAb 6D7 was purified from the ascites fluid by the caprylic-acid–ammonium-sulfate precipitation method. As shown in the Figure 3(a,b), the subclass of the McAb was identified as IgG2b, and 6D7 had high affinity constant with $6.3 \times 10^9$ L/mol. The results in Figure 3(c) showed that the McAb is highly specific to DEX, where the CR of 6D7 to DEX analogues tested was <5%. As shown in Figure 3(d), a standard curve was built for DEX detection and the equation is $y = 0.01706 + 1.6682/(1 + (x/0.09518)^{1.35289})$, with a correlation coefficient ($R^2$) of 0.9988. From above equation, IC_{10}, IC_{20}, IC_{50}, and IC_{80} of the McAb to DEX was 0.017, 0.034, 0.095, and 0.265 ng/mL, respectively. Thus, the linear range to detect DEX was 0.034–0.265 ng/mL and the LOD was 0.017 ng/mL. Because of the high affinity and specificity, 6D7 would be chosen for further experiments.

CG immunoassay establishment

The PBS spiked a series of DEX concentrations of 0, 0.05, 0.1, 0.2, and 0.3 ng/mL was prepared and incubated with the McAb labeled CG to ensure sufficient reaction of antigen and antibody. The cut-off value was defined as the concentration of DEX at the time only C line was visible on strip and would be used to assess the sensitivity of the CG strip. As shown in Figure 4(a), when there was no DEX present, both the T line and C line showed red color. As the concentration of DEX risen, the color of T line became lighter and lighter. Until the concentration reached 0.3 ng/mL, only C line could be observed. Thus, the cut-off value of this strip method was 0.3 ng/mL in PBS.

Detection of DEX in milk samples by strip assay

In fact, milk samples were different to PBS due to its matrix effects. In order to validate whether the assay could be used for detecting DEX in milk samples, a series of DEX were spiked into blank milk that were purchased in the local markets and confirmed by LC-MS. A series of DEX concentrations of 0, 0.05, 0.1, 0.2, 0.3, and 0.5 ng/mL were spiked in milk samples and analyzed by the strip assay. As shown in Figure 4(b), the cut-off values were 0.5 ng/mL in milk sample. The test results of CG assay can be converted into

![Figure 2](image_url). The UV–VIS spectroscopy of DEX, DEX–HS, protein and conjugates: (a) confirmation of immunogen (DEX–HS–BSA) and (b) confirmation of coating antigen (DEX–HS–OVA).
numeric values of DEX content. A negative result occurred when the concentration of DEX was \( \leq 0.05 \) ng/mL; a weakly positive result occurred when the concentration of DEX was 0.05–0.5 ng/mL; and positive results occurred when the concentration of DEX was \( \geq 0.5 \) ng/mL. So this detection method could be used to investigate the presence of DEX residue in milk. However, compared with results in PBS, the visual cut-off values were

![Figure 3](image.png)

**Figure 3.** Characterization of McAb 6D7. (a) Isotype determination of 6D7; (b) Affinity result of 6D7; (c) Cross-reaction of 6D7; and (d) Standard curve for DEX detection.

![Figure 4](image.png)

**Figure 4.** Image of detection DEX by CG strip in PBS (a) and milk sample (b). (a) 1 = 0 ng/mL, 2 = 0.05 ng/mL, 3 = 0.1 ng/mL, 4 = 0.2 ng/mL, and 5 = 0.3 ng/mL; Cut-off value was 0.3 ng/mL. (b) 1 = 0 ng/mL, 2 = 0.05 ng/mL, 3 = 0.1 ng/mL, 4 = 0.2 ng/mL, 5 = 0.3 ng/mL, and 6 = 0.5 ng/mL; Cut-off value was 0.5 ng/mL.
higher, and the color of C line was lighter. The reason for this phenomenon may be that complex compositions in milk influenced the reaction of antibody and antigen.

**Analysis of DEX in milk samples**

To validate the accuracy of CG assay, a comparison experiment between ic-ELISA and CG assay was carried out. As shown in Table 1, with the ic-ELISA, the recovery rates ranged from 92.4% to 102.8% for DEX and the coefficients of variation (CV) ranged from 4.43% to 10.82%. And with the strip assay, DEX can be semi-quantitatively analyzed and all detection results were acquired within 10 min. The results obtained from ic-ELISA and the CG strip assay were consistent for detection of DEX in milk samples.

**Conclusion**

In this study, DEX was derived by the succinic anhydride method and a carboxyl group was introduced to allow protein coupling. Then a sensitive anti-DEX McAb was obtained by immunization and cell fusion. Anti-DEX McAb with high affinity of $6.3 \times 10^9$ L/mol and high specificity to DEX was successfully applied to establish CG immunoassay for detecting DEX in PBS and milk. The cut-off values were different in PBS and milk due to matrix interference. In spiked samples and a recovery test, the recovery rates of ic-ELISA ranged from 92.4% to 102.8% for DEX, and the results of strip assay had a strong agreement with ic-ELISA. Thus, the CG assay could be used as a screening method for detecting DEX in real samples.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was funded by the Key Programs from MOST [grant numbers 2016YFD0401101, 2016YFF0202300], and grants from the Natural Science Foundation of Jiangsu Province, MOF and MOE [grant numbers BE2016307, BK20140003, BX20151038, BE2013613, BE2013611].

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