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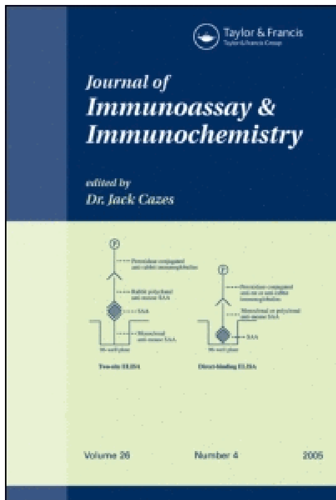

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Development of Immunochromatographic Assay for the On-site Detection of Salbutamol

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Abstract: Salbutamol, one of the β-agonists, is misused as a growth promoter in meat producing animals. In-house synthesized colloidal gold was conjugated with the polyclonal anti-salbutamol antibodies. A rapid immunochromatographic assay was developed in a competitive format. The salbutamol-BSA conjugate and goat anti-rabbit IgG were immobilized on a nitrocellulose membrane as test and control lines, respectively. The color intensity of a purple test line was inversely proportional to the amount of salbutamol presenting in the samples. The sensitivity was estimated to be about 80 ng/mL of salbutamol in PBS. The method can be useful as an "on-site" screening procedure for detection of salbutamol.

Keywords: Antibodies, β-agonists, ELISA, Gold colloid, Immunochromatographic strip test, Salbutamol

INTRODUCTION

Salbutamol, a synthesized β-adrenergic agonist, has been used in the treatment of asthma and applied as a tocolytic agent in human, as well as in veterinary medicine.[1] Such, β-agonists are also used illegally in

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animal husbandry to enhance the growth rate and improve the lean-meat content of meat-producing animals, with the resulting substantially increased profit.[2,3] At high doses, these drugs have been found to cause weight gain in animals together with a repartition between muscle and fatty tissue, with the resulting increased carcass value. Accumulation of β-agonist residues in edible tissues of treated animals results in adverse effects on the cardiovascular and central nervous system of consumers.[4–6] Various toxic effects have been ascribed to the ingestion of liver and meat containing β-agonist residues.[7–9] A number of analytical methods have been developed for the determination of β-agonist residues in animal tissue and body fluids such as HPLC,[10–12] GC-MS,[13–16] and LC-MS-MS.[17]

These methods require extensive sample clean-up and personnel with professional training to operate the sophisticated instruments. Certain screening methods for β-agonist detection, based on enzyme- or radio-immunoassay, have been described by a number of workers.[18–21] These methods provide the advantage of portability, high throughput, and user-friendly analysis. However, the assay time required for this technique is longer than one hour, and the assay requires a well-trained technician and special equipment in the laboratory and, thus, is not suitable for detection on-site. Several groups have developed "on site" screening tests for detection of β-agonist residues.[22–24] Although these methods can be used at the place of sampling, all are based on an enzyme-detection system which has some tedious steps such as incubation, washing and development of enzymatic reactions during signal generation. More recently, colloidal gold-based immunochromatographic assays have been used as "on site" screening tests. Colloidal gold has advantages as a detector in the assay compared to enzymatic detection due to its easier visualization and higher stability. These characteristics significantly shorten the analysis time and make it very convenient for "on site" testing.

Herein, we describe the preparation and characterization of salbutamol-BSA conjugate, anti-salbutamol polyclonal antibodies and an antibody-colloidal gold probe. These reagents were used to develop a colloidal gold-based immunochromatographic assay, which can be used as a rapid and simple screening method for the detection of salbutamol in veterinary fields.

EXPERIMENTAL

Chemicals and Reagents

Salbutamol free base, clenbuterol, succinic anhydride, dioxane, isobutyl chloroformate, triethyllamine, chloroauric acid ($HAuCl_4 \cdot 3H_2O$) and

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bovine serum albumin (BSA) were purchased from Sigma, USA. HRP-swine anti-rabbit immunoglobulin conjugate was obtained from Zymed, CA, USA. Goat anti-rabbit IgG was obtained from Aristabiologicals Inc., USA. Chloramphenicol-BSA was produced in our laboratory.[25] High-flow nitrocellulose membranes, glass fibers and absorption pads were obtained from Schleicher and Schuell (Dassel, Germany).

Preparation of Salbutamol Succinate

The salbutamol succinate was prepared as described by Beaulieu[18] with minor modifications. Briefly, salbutamol free base (150 mg, 0.62 mmol) was dissolved in 6 mL of absolute methanol. After sonicating for 5 minutes, the solvent was removed under vacuum condition using a rotatory evaporator (Büchi, Switzerland). The yellow oily residue was re-dissolved in 6 mL of absolute ethanol at 0°C. While stirring, succinic anhydride (67 mg, 0.67 mmol) was added. After 5 minutes, a cloudy white suspension appeared and the mixture was stirred at the ambient temperature for 16 hours. The formation of the salbutamol succinate was monitored by thin layer chromatography (ethyl acetate-methanol-triethylamine 60:39:1, R_f salbutamol = 0.22; R_f salbutamol succinate = 0.13). Then, the suspension was centrifuged at 3,000 rpm for 15 minutes, and the solid phase was washed three times with absolute ethanol and dried. The melting point of the salbutamol succinate was approximately 142–143°C, similar to those reported by Beaulieu et al.[18] and Degand et al.[26] The following ^{1}H NMR (400 MHz, CD_3OD) was produced: δ 7.34 (Brd, 1H), 7.25 (Brdd, $J = 8.4$ Hz, 1H), 6.83 (d, $J = 8.4$ Hz, 1H), 5.18 (s, 1H), 4.82 (m, 1H), 3.09 (m, 3H), 2.63 (m, 4H), 1.39 (s, 9H). Salbutamol succinate was analyzed by LC–ESI-MS. The most intense product ion $[M+1]^+$ of salbutamol succinate [$C_{17}H_{26}NO_6$] was observed at m/z 340.13, which was consistent with the molecular weight calculated from the formula (339.37).

Coupling of Salbutamol Succinate to BSA

Salbutamol-BSA conjugate (SALB-BSA) used as an immunogen was prepared according to the mix anhydride method.[27] Salbutamol succinate (40 mg, 0.12 mmol) was dissolved in a mixture of dioxane-water- triethylamine (25/3/0.3 v/v/v). The solution was stirred for 30 minutes at room temperature. Then, isobutyl chloroformate (30 μL, 0.23 mmol) was added and the solution was stirred continuously for 2 hours. The solution was added to the diluted BSA (86 mg in 25 mL distilled water). The resultant

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solution was stirred overnight at 4°C and dialyzed in phosphate buffered saline (pH 7.2) at 4°C for 2 days, with four changes of buffer. The dialyzed solution of SALB-BSA was lyophilized and kept at −20°C until use.

Preparation of Polyclonal Antibodies

SALB-BSA was prepared in an emulsion of sterile PBS with complete Freund's adjuvant or incomplete Freund's adjuvant in a ratio of 1:1. Preimmunized serum was collected from an albino New Zealand rabbit before subcutaneous multi-site injections. The rabbit was immunized with 1 mL of the emulsion (1.0 mg of SALB-BSA in complete Freund's adjuvant/injection). Four booster injections (1.0 mg of SALB-BSA in incomplete Freund's adjuvant/injection) were given every week after the first injection. Blood samples were collected before each injection, and titers of the antisera were determined by indirect ELISA. The hyperimmune serum obtained 2 weeks after the last injection was collected and precipitated with saturated $(NH_4)_2SO_4$. The obtained immunoglobulin fraction was dialyzed against 5 mM sodium borax buffer pH 9.0.

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Determination of Anti-Salbutamol Antibody Responses in a Rabbit by Competitive Indirect ELISA

Each well of a microtiter plate was coated with 50 μL of 10 μg/mL of either SALB-BSA or unconjugated BSA in carbonate/bicarbonate buffer, pH 9.6 at 4°C for 18 hours. Unbound antigens were removed from the plate by five times washing with washing buffer (0.05% Tween 20 in PBS pH 7.2). The coated plate was filled with 200 μL/well of the blocking solution (2% skim milk in PBS, pH 7.2) and incubated at room temperature for 2 hours to block unoccupied sites on the plate. After washing five times with washing buffer, the six sets of rabbit sera were serially two-fold diluted in 2% BSA/PBS, and then added into the wells (50 μL/well). The plate was then incubated for 1 hour and washed 5 times with the same reagent. To trace antibody binding, 50 μL of HRP-swine anti-rabbit immunoglobulin conjugate was added into each well. After incubation for 1 hour and five times washing, TMB/H_2O_2 substrate-buffered solution was applied and the plate was incubated in the dark for a short duration. The enzymatic reaction was stopped with 100 μL of 1 N HCl and the absorbance was measured at a wavelength of 450 nm using a microtiter plate reader (TECAN, Austria).

Western Immunoblotting for Verifying the SALB-BSA Immunized Rabbit Serum

SALB-BSA, unconjugated BSA and chloramphenicol-BSA were electrophoretically separated by SDS-PAGE under reducing condition in 12% polyacrylamide separating gel. For Western immunoblotting, the separated polypeptides were electroblotted to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in PBS at 4°C for 18 hours. Subsequently, 1 μg/mL of the polyclonal anti-salbutamol antibody in 5% BSA/PBS was applied to the blocked membrane on a shaking platform for 1 hour at room temperature. The unbound antibody was washed out and then incubated with HRP-swine anti-rabbit immunoglobulin conjugate (1:5,000 in 5% skim milk/PBS) for 1 hour at room temperature. After five times washing with 0.05% Tween 20 in PBS (pH 7.2), the immunoreactive bands were developed using TMB/H_2O_2 immunoblotting substrate solution, and then exposed to Kodak X-ray film. The molecular size of each reactive band was calculated relative to standard proteins.

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Preparation of Polyclonal Anti-Salbutamol Antibodies-Colloidal Gold Conjugate

Colloidal gold with a mean diameter of 15 nm was synthesized by reduction of $HAuCl_4$ with sodium citrate as described previously by Pattarawarapan.[28] The polyclonal anti-salbutamol antibodies were conjugated with colloidal gold as the detection probe as follows. Briefly, colloidal gold solution was adjusted to pH 7.0 with 0.2 M potassium carbonate. Nine millilitres of polyclonal antibodies at the optimum concentration of 0.25 mg/mL were incubated with 90 mL of colloidal gold solution for 30 minutes at room temperature. Then, 7.2 mL of 5% BSA in 5 mM NaCl solution was added to block the un-reacted sites of gold surfaces. The mixture was incubated at room temperature for another 10 minutes and then centrifuged at 15,000 rpm for 30 minutes to remove the unconjugated antibody and BSA in the solution. The pellet was suspended in phosphate gold diluent buffer (1% BSA in 49 mM Na_2HPO_4). The polyclonal anti-salbutamol antibodies-colloidal gold conjugate solution (anti-SALB-CGC) was further characterized by UV-Vis spectroscopy.

Characterization of Colloidal Gold Conjugate by UV-Vis Spectroscopic Studies

The formation of anti-SALB-CGC was monitored by UV-vis spectroscopy (λ 200–700 nm) using a double-beam spectrophotometer

(Shimadzu, Japan) at 1 nm. The gold solutions were monitored immediately after addition of antibody and BSA. To ensure removal of unconjugated protein, the gold conjugate solution was centrifuged and the pellet redispersed in appropriate buffer, and the characteristic of the spectrum were monitored.

Preparation of an Immunochromatographic (IC) Test Strip

An immunochromatographic test strip (IC-test strip) consists of four components: sample application pad, conjugate releasing pad, analytical nitrocellulose membrane, and absorbent pad. An IC-test strip was constructed as follows anti-SALB-CGC (O.D. of 40 at λ 580 nm) was sprayed onto the conjugate releasing pad using an IsoFlow dispenser (Imagene Technology, USA). The nitrocellulose membrane was laminated onto a plastic support by a Precision Laminator (Zeta Corporation, Korea). SALB-BSA antigen at 0.5 mg/mL and goat anti-rabbit IgG at 0.12 mg/mL in PBS were jetted onto a laminated nitrocellulose membrane at two separate zones, referred to as the test line and control line. Subsequently, the sprayed conjugate pad and jetted membrane were incubated for 4 hours at 37°C and then dried in a desicator at room temperature. After drying, the components of the strip test (the sample application pad, the sprayed conjugate releasing pad, the jetted nitrocellulose membrane, and the absorbent pad) were assembled and then cut into individual strips (4.0 mm/strip) using an A-Point Programmable Guillotine Cutter (A-Point Technologies Inc., NJ).

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Principle and Procedure for Analysis of Salbutamol Standard Solution

Analysis of salbutamol by the IC-test strip was carried out using salbutamol standards prepared by diluting a stock solution with 10% methanol in PBS. In principle, an IC strip test is immersed vertically into 100 μL of a sample for 10 sec and then placed horizontally on a non-absorbent flat surface. The liquid sample will migrate by capillary diffusion through the conjugate pad, rehydrating anti-SALB-CGC. When the solution moves upward to the test line zone, the anti-SALB-CGC will be trapped by the immobilized SALB-BSA on the membrane forming a clear red test line. The excess molecules of the anti-SALB-CGC migrate further and are trapped by goat anti-rabbit IgG antibody, forming the control line. The whole analysis is usually completed within 10 minutes, and the result can be read by the naked eye. A negative result is indicated by the appearance of

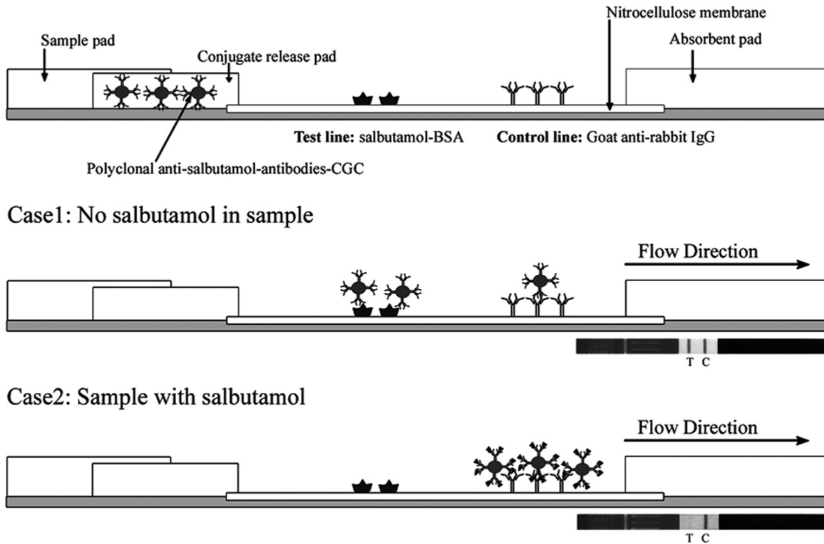

Figure 1. Configuration of the immunochromatographic test strip. The schematic diagram shows the areas where anti-SALB-CGC, SALB-BSA and goat anti-rabbit IgG are immobilized. The reactions that occur on the IC-test strip in the absence of salbutamol (Case 1), and in the presence of salbutamol (Case 2) are shown. A red-purple color appears at the test line and/or control line, depending on the presence of salbutamol in sample.

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two red lines in the control and test regions. In the presence of salbutamol, the salbutamol will compete with the immobilized SALB-BSA on the test line to bind the limited amount of the anti-SALB-CGC. The more salbutamol present in the sample, the more effectively it will be able to block the capture of the anti-SALB-CGC. Therefore, an increase in the amount of salbutamol in the sample solution will result in a decrease in the signal in the test line zone. A positive result is indicated by the appearance of only one line in the control region. A schematic description of the IC-test strip is shown in Fig. 1.

Cross-Reactivity of the Immunochromatographic Strip Test

Clenbuterol and chloramphenicol were diluted at 10, 100, 1,000, 10,000, 100,000, and 1,000,000 ng/mL and then separately assayed by IC-test strips for evaluating cross reactivity.

RESULTS

Synthesis of Salbutamol-BSA Conjugate

Salbutamol succinate was synthesized and then conjugated with BSA molecule. Conjugation of salbutamol to BSA was verified by Indirect ELISA and Western immunoblotting (data not shown).

Validation of Polyclonal Antibodies

SALB-BSA was synthesized and used as the immunogen to trigger an effective immune response against SALB-BSA in the rabbit. Indirect ELISA was carried out for monitoring the antibodies against salbutamol. A high titer at 1:120,000 was observed after the fifth immunization. The obtained hyperimmune serum was precipitated using 33.3% saturated ammonium sulfate to yield immunoglobulin fraction.

To further assure the specificity of the binding, Western immunoblotting was performed using the polyclonal anti-salbutamol antibodies as a capture antibody to probe the proteins blotted onto PVDF membrane (Fig. 2). SALB-BSA (Lanes 1, 4, and 5), unconjugated BSA (Lane 2) and Chloramphenicol-BSA (Lane 3) were separated by SDS-PAGE under the reducing condition and blotted onto PVDF membrane. Lanes 1, 2 and 3 were probed with the polyclonal anti-salbutamol antibodies (1 μg/mL). A positive signal was only observed in lane 1, indicating the recognition of polyclonal anti-salbutamol antibodies. No positive signal was observed in lanes 2 and 3. This result suggests that the anti-BSA antibodies were neutralized by BSA contained in the diluent solution (5% BSA/PBS). In addition, the antibodies recognizing the succinic linkers that appear in both SALB-BSA and chloramphenicol-BSA molecule was not provoked. Lane 4 was incubated with the mixture of salbutamol 100 ng/mL and the polyclonal anti-salbutamol antibodies (1 μg/mL). The positive signal of SALB-BSA (lane 4) was dramatically decreased compared to that of lane 1. This result confirmed the specificity to the salbutamol molecule of the polyclonal anti-salbutamol antibodies. The conjugate control (Lane 5), in which the primary antibody was omitted during the first incubation solution, followed by addition of HRP-swine anti-rabbit immunoglobulin conjugate, showed no immunoreactive band.

Characterization of Colloidal Gold Conjugate by UV-Vis Spectroscopic Studies

The polyclonal anti-salbutamol antibodies were directly added to the colloidal gold solution to form conjugate, and the presence of antibody

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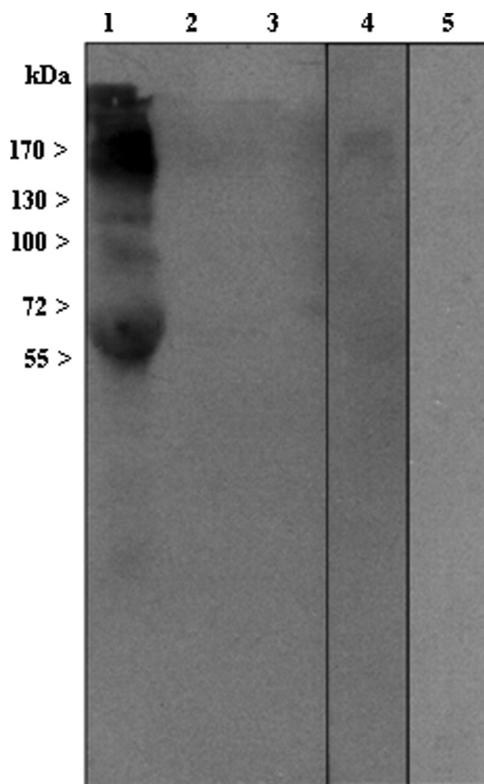

Figure 2. Analysis of polyclonal anti-salbutamol antibodies by Western immunoblotting technique. Lanes 1, 4 and 5 were SALB-BSA. Lane 2 was unconjugated BSA. Lane 3 was chloramphenicol-BSA. All proteins were separated under reducing conditions. Lanes 1, 2 and 3 were probed with the polyclonal anti-salbutamol antibodies (1 μg/mL). Lane 4 was probed with the mixture of salbutamol (100 ng/mL) and the polyclonal anti-salbutamol antibodies. Lane 5 was the conjugate control in which the primary antibody was omitted. The HRP-swine anti-rabbit immunoglobulin conjugate was applied to all lanes. Molecular weight markers (kDa) are indicated.

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on the gold surface was characterized using UV-Vis spectroscopy (Fig. 3). Spectra of the colloidal gold solution were recorded without proteins (Curve *a*), immediately after the addition of both antibody (Curve *b*) and BSA (Curve *c*), and after centrifugation and resuspension with 2 mL of 5 mM sodium borax buffer (Curve *d*). The peak at ~519 nm in curve *a* is due to the surface plasmon resonance of colloidal gold particles. After the addition of the polyclonal anti-salbutamol antibodies, curve *b* showed a red shift when compared with curve *a*. The red shift

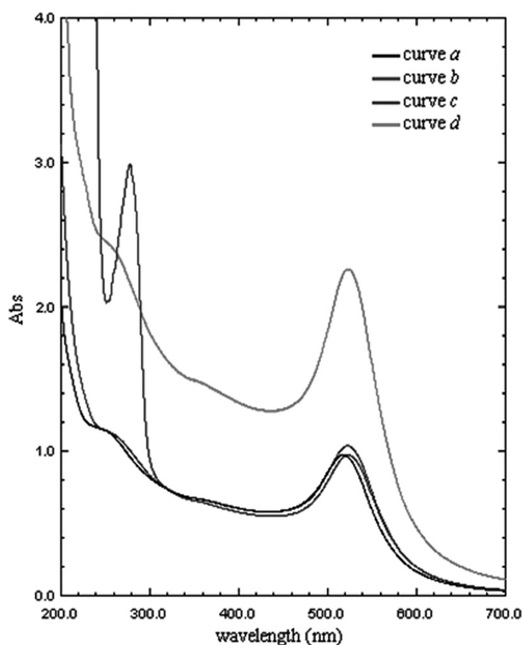

Figure 3. UV-vis spectra of the colloidal gold and gold conjugates. Curve *a* is a spectrum of colloidal gold solution; ([Au] is about 2.5×10^{-4} mol/L). Curve *b* is a spectrum of gold conjugate solution in which the polyclonal anti-salbutamol antibodies were immediately added to gold solution; ([Au] is about 2.5×10^{-4} mol L^{-1}). After incubating the antibodies with gold solution, BSA was then applied to gold conjugate solution; ([Au] is about 2.5×10^{-4} mol/L) (Curve c). Curve *d* is anti-SALB-CGC after centrifuging and resuspending with 2 mL of 5 mM sodium borax buffer [Au is about 6.25×10^{-4} mol/L].

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phenomenon occurred due to the interaction of the antibody with the colloidal gold particles. Curve *c*, which was recorded immediately after adding BSA, displayed the red shift, as well, and a new peak appeared at ~280 nm, which corresponded to a protein absorption band. After the centrifugation and resuspension of the gold conjugate, the intensity of the plasmon band increased due to the enhanced colloidal gold concentration (curve *d*). However, the intensity of absorption at 280 nm was diminished, indicating that the excess unconjugated proteins in the solution were removed during the washing step.

Analysis of Salbutamol Standard Solution with the IC-Test Strips

Immunochromatographic test strips were constructed using SALB-BSA, goat anti rabbit IgG, and anti-SALB-CGC (OD 40 at λ 580 nm) as a

test line, a control line, and a detector, respectively. To evaluate the performance of IC-test strips, various concentrations of salbutamol spiked in PBS were assessed. The representative results of 10 repeated experiments are shown in Fig. 4. In the absence of salbutamol, the binding of anti-SALB–CGC with the immobilized SALB–BSA onto a nitrocellulose membrane appeared as an intense red-purple band at the test line. In the presence of salbutamol in the sample solution, the color intensity of the test line gradually decreased with increasing concentration of salbutamol, and disappeared completely at 80 ng/mL of salbutamol in PBS. In addition, concentrations as high as 1,000 ng/mL did not affect the color intensity of the control line in which the binding of anti-SALB-CGC with the goat anti- rabbit IgG took place.

In order to evaluate the preliminary practicability of IC test strip in real samples, the pool swine urine spiked with various concentrations of salbutamol, clenbuterol or chloramphenicol were determined by IC-test strips. The results showed no effect on sensitivity and specificity in comparison with PBS.

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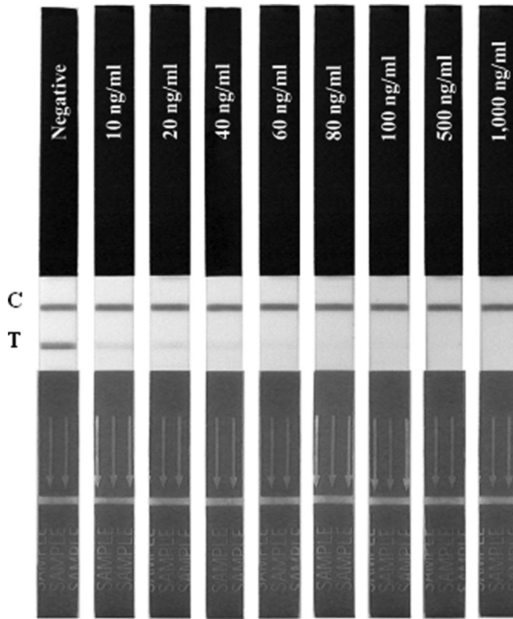

Figure 4. Immunochromatographic test strip results for salbutamol at concentrations of 0, 10, 20, 40, 60, 80, 100, 500, and 1,000 ng/mL in PBS. C = control line; T = test line

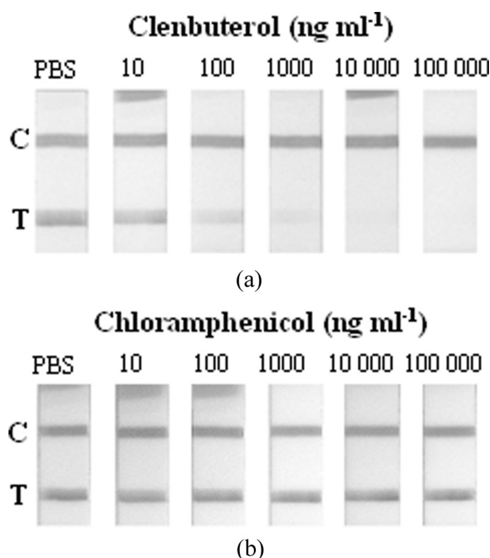

Figure 5. Cross-reactivity study of immunochromatographic test strips. (a) A serial dilution of clenbuterol ranging from 0–100,000 ng/mL. (b) A serial dilution of chloramphenicol ranging from 0–100,000 ng/mL.

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Cross Reactivity Study of IC-Test Strips

In the cross-reactivity study, a structurally related compound of salbutamol, clenbuterol and an unrelated compound, chloramphenicol, were used to probe probable interference with the strip test. As shown in Fig. 5, the intensity of the test line band slightly faded when assaying with the test solution containing clenbuterol at 100 ng/mL. Moreover, the color of the test line band was found to be almost gone when the test solution contains clenbuterol at 10,000 ng/mL. In contrast, chloramphenicol showed no competition with anti-SABL-CGC conjugate.

DISCUSSION

A simple and fast qualitative screening test that can be performed at the place of sampling (markets, farms or slaughter houses) is a powerful tool to achieve the effective surveillance of the illegal use of β-agonists. The "on site" screening tests based on enzyme-detection system for detection of β-agonist residues have been developed by several groups. Ploum et al.[23] have reported the preliminary results of the development of a test strip assay based on a competitive enzyme immunoassay for the screening

of urine samples for the presence of clenbuterol. The positive samples could be distinguished above 5 ng/mL level. Haasnoot et al.[22] have modified the competitive ELISA which can be performed in polystyrene tubes for "on site" testing purpose. Bovine urine samples with a level of 3 ng/mL of clenbuterol and higher were found positive with their assay. Vanoosthuyze et al.[24] have also developed the test strip immunoassay which can be performed on the spot for the determination of β-agonists in urine. They demonstrated that the extraction on Empore membranes provided a fast and simple clean-up of the urines. Their assay had a visual detection limit for clenbuterol of 1 ng/mL of urine and showed good sensitivity for salbutamol. However, enzyme-detection system has some fussy operations such as incubation, washing and enzymatic reactions during signal generation. In recent years, colloidal gold-based immunochromatographic assay is wildly used as "on site" screening tests due to the simplicity of the procedures and the rapidity of the result. In the veterinary fields, several studies on immunochromatographic assay for monitoring various analytes, i.e., salinomycin,[29] and neomycin[30] were reported. In addition, the utility of immunochromatographic assay for analysis of clenbuterol has been demonstrated by Zhang et al.[31] Herein, an immunochromatographic assay based on polyclonal anti-salbutamol antibodies has been successfully developed for use in the detection of salbutamol. SALB-BSA was synthesized and used as the immunogen to trigger an effective immune response against SALB-BSA in the rabbit. The rabbit polyclonal anti-salbutamol antibodies were raised after immunized with SALB-BSA immunogen. After precipitation, the polyclonal anti-salbutamol antibodies were verified by Western immunoblotting. Results showed the specificity to the salbutamol molecule of the polyclonal anti-salbutamol antibodies. Colloidal gold solution was synthesized by the Turkevieh method[32] using sodium citrate as a reducing agent. The polyclonal anti-salbutamol antibodies were directly added to the colloidal gold solution to form conjugates. The formation of anti-SALB-CGC was demonstrated by UV-Visible light absorption spectra. Immunochromatographic test strip was constructed using SALB-BSA, goat anti rabbit IgG, and anti-SALB-CGC as a test line, a control line, and a detector, respectively. Preliminary result showed that the IC-test strip was possible to detect the presence of salbutamol at a concentration as low as 80 ng/mL within 10 min by visual observation. The anti-SALB-CGC showed little cross-reactivity with clenbuterol whereas no cross reactivity with chloramphenicol. In addition, the effect of swine urine matrix on IC-test strip was also validated. The pool swine urine was spiked with various concentrations of salbutamol, clenbuterol or chloramphenicol. Interestingly, there was no effect on sensitivity and specificity in comparison with PBS. Practically, the test strip should be directly used at the livestock or slaughterhouse as a non-invasive procedure.

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In conclusion, the results indicate that we successfully developed an immunochromatographic test strip for the rapid detection of salbutamol. The assay is a potentially useful tool for screening salbutamol residues due to its rapid and simple procedure, one that does not require sophisticated instruments. Therefore, the developed technique should assist in detecting the salbutamol contaminated meat before it reaches the consumer market. Although the preliminary results suggest that the IC-test strip is suitable for practical uses, the sensitivity and specificity in animal samples need to be further quantified; this will be the goal of future research.

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