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Novel ractopamine–protein carrier conjugation and its application to the lateral flow strip test for ractopamine detection in animal feed*#

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Abstract: In this work, a novel conjugate of ractopamine and bovine serum albumin (RAC–BSA) has been developed via the Mannich reaction, with a mole coupling ratio for RAC–BSA of 9:1. The proposed conjugation method provides a simple and one-step method with the use of fewer reagents compared with other conjugation methods for competitive immunoassays. RAC–BSA conjugation was used to fabricate a competitive lateral flow strip test for RAC detection in animal feed. For sample preparation, RAC was spiked in swine feed purchased from the local markets in Thailand, and methanol and running buffer at a volume ratio of 10:90 was used as extraction buffer. The procedures for sample preparation were completed within 25 min. Under optimal conditions, the limit of detection (LOD), assessed by the naked eye within 5 min, was found to be 1 ng/g. A semi-quantitative analysis was also conducted using a smart phone and computer software, with a linearity of 0.075–0.750 ng/g, calculated LOD of 0.10 ng/g, calculated limit of quantitation of 0.33 ng/g, and good correlation of 0.992. The recoveries were found in the range of 96.4%–103.7% with a relative standard deviation of 2.5%–3.6% for intra- and inter-assays. Comparison of the results obtained by the strip test with those obtained by enzyme-linked immunosorbent assay had a good agreement in terms of accuracy. Furthermore, this strip test exhibited highly specific RAC detection without cross reactivity with related compounds. Therefore, the RAC–BSA conjugation via the Mannich reaction can be accepted as a one-step and easy conjugation method and applied to the competitive lateral flow strip test.

Key words: Ractopamine; Conjugate of ractopamine and bovine serum albumin (RAC–BSA); Mannich reaction; Lateral flow strip test; Feed additive
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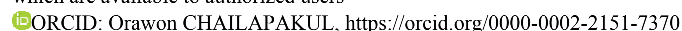

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1 Introduction

Ractopamine (RAC, $C_{18}H_{23}NO_3$, molecular weight (MW) 301.386 g/mol) is a synthetic organic compound in the β-agonist group that shares a common chemical structure with the compound classified as phenethanolamine (Shishani et al., 2003; Zhang et al., 2009a; Wang et al., 2010). β-Agonists bind to β-receptors on fat cells to activate several enzymes in the pathway that leads to a decreased rate of lipid synthesis and storage and increased lipid mobilization in the cells (He et al., 2007; Shen and He, 2007; Gao et al., 2014). This group of compounds can also bind to β-receptors on skeletal muscle cells for the activation of protein kinases, leading to the enhancement of muscle protein synthesis and muscle growth (Liu et al., 2009; Li et al., 2015; Liang et al., 2016). Therefore, β-agonists, particularly RAC, are widely used as feed additives for cattle and swine to reduce body fats and increase muscle tissues. Initially, the appearance of RAC residues in the edible tissue of animals due to the feeding of the animals with a feed supplement containing RAC has been observed. Based on a previous report on feed supplements containing RAC (Patience et al., 2009), 5 μg/g of RAC in the feed supplement affected the growth performance in swine and ultimately the pork quality, and the maximum residue limits (MRLs) in veterinary food for RAC from the Codex Alimentarius Commission (Codex) are in the range of 10–90 ng/g, depending on the type of edible tissues. Furthermore, the overall effects of RAC on human health include cardiac stimulation, which causes an increased heart rate and systemic dilation of blood vessels (Shelver and Smith, 2002; Smith and Shelver, 2002; Du et al., 2014). This has led to an import prohibition of RAC-treated meat in many countries, notably Europe and China (Niño et al., 2017). Therefore, the importance of detection methods for RAC in animal feed is to help detect and thereby control the appearance of RAC in edible tissues.

Currently, an immunoassay has been widely used as a detection method because of its high specificity and sensitivity for RAC (Shelver and Smith, 2003; Han et al., 2013; Lei et al., 2013). The conventional immunoassay—the enzyme-linked immunosorbent assay (ELISA)—has several disadvantages, such as complicated steps, prolonged time, and the requirement of expensive instrument (Holme and Peck, 1998; Berlina et al., 2017). A lateral flow strip test has been proposed as a portable device to overcome the drawbacks of the conventional ELISA in terms of the use of fewer solutions, lower costs, rapid assay, ease of use and disposability (Teerinen et al., 2014; Tang et al., 2016). In the case of RAC detection by immunoassay, a competitive format, which involves competition between the free RAC in samples and immobilized RAC that is spiked into the samples to bind with an antibody, is normally used owing to the smaller size of RAC molecules (Haasnoot et al., 1994; Shelver and Smith, 2000; Liu et al., 2014). In addition, RAC must be conjugated to protein carriers such as bovine serum albumin (BSA) (Ren et al., 2014; Shi et al., 2015; Hu et al., 2017) and ovalbumin (OVA) (Zhang et al., 2009b; Li et al., 2010; Dong et al., 2012) to form an RAC–protein carrier for immobilization on the substrate surface. Therefore, the conjugation method of the RAC–protein carrier is extremely important in the immunoassay for RAC. A previous report on RAC–protein carrier conjugation by changing the functional group of RAC to a carboxylic group (Buakeaw et al., 2016) states multiple steps and the use of various types of chemicals, particularly pyridine, to dissolve chemicals in the conjugation pathways, leading to the requirement of an additional step to evaporate pyridine. Another method, involving the reductive reaction of octopamine to form an RAC hapten before its conjugation to the protein carrier using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide as coupling agents, has been reported and describes complicated steps and the use of several types of chemicals (Gu et al., 2016). Owing to the disadvantages of the previous conjugation methods, this simple and fast method with less chemical utilization is an interesting approach as an alternative method for RAC–protein carrier conjugation.

Previously, some researchers reported the synthesis of haptens such as aflatoxin B1-cationized BSA (Zhou et al., 2007) and bisphenol A-cationized BSA (Feng et al., 2009) via the Mannich reaction. The Mannich reaction occurs when active hydrogen-containing compounds condense with aldehydes, particularly formaldehyde, and an amine under weak acidity (Hermanson, 2008). The hapten synthesis via the Mannich reaction exhibits good immunological properties, simple coupling steps, stable alkylamine linkage, and the use of fewer types of chemicals

(Zhou et al., 2007; Feng et al., 2009). Literature reviews reveal that there is no report on the use of the Mannich reaction for conjugation between RAC and protein carrier. Therefore, the Mannich reaction interestingly acts as a novel method for RAC–protein carrier conjugation.

In this work, we aim to construct a method for conjugating RAC with a protein carrier in a single step involving simplicity, rapidity, and the requirement of fewer reagents based on the Mannich reaction. The monoclonal antibody (mAb) against RAC produced by our lab (Buakeaw et al., 2016) and the RAC–protein carrier were applied to fabricate the lateral flow strip test for RAC detection. The immunological response of the novel RAC–protein carrier conjugate and the sensitivity for RAC detection in animal feed (using the strip test) were visually assessed by the naked eye (within 5 min) as a qualitative analysis, exhibiting the advantages of a simple and rapid test. A semi-quantitative analysis was also performed using a simple instrument through a smart phone equipped with computer software (ImageJ software). In addition, the strip test was applied to quantifying the amounts of RAC in animal feed to validate its performance with the conventional method (ELISA).

2 Materials and methods

2.1 Chemicals and materials

RAC, BSA, glycine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, pyridine, *tris*(hydroxymethyl) aminomethane, succinic anhydride, *N*,*N*'-disuccinimidyl carbonate, methanol, Tween 20, sucrose, and formaldehyde were purchased from Sigma Aldrich (St. Louis, MO, USA). Goat-antimouse immunoglobulin G (GAM) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Gold nanoparticles (AuNPs, 20 nm) were purchased from Serve Science Co., Ltd. (Chatuchak, BKK, Thailand). Pierce™ protein assay kit was purchased from ThermoFisher Scientific (Waltham, MA, USA). All solutions were prepared using 18 Ω milli-Q water. Protein G sepharose, nitrocellulose membrane (NCM, AE99), glass fiber membrane (Standard 17 as sample pad and GF33 as conjugate pad), and absorbent pad (CF7) were purchased from Whatman-GE Healthcares (Pittburgh, PA, USA).

2.2 Conjugation of RAC and protein carrier

The RAC–BSA conjugation was performed via the Mannich reaction with slight modification (Hermanson, 2008). BSA dissolved in 0.1 mol/L 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 4.7) containing 0.15 mol/L NaCl was initially mixed with RAC followed by the addition of 37% (v/v) formaldehyde. Subsequently, the mixture solution was stirred and incubated in the dark at room temperature overnight. Finally, the mixture solution of RAC–BSA was dialyzed using 0.01 mol/L phosphate-buffered saline (PBS; pH 7.4) and stored at −20 °C until use.

2.3 Calculation of conjugate molar ratio of RAC and BSA

To confirm the conjugation of RAC–BSA, the absorbance of RAC, BSA, and RAC–BSA was measured using an ultraviolet-visible (UV-vis) spectrophotometer, and the conjugate molar ratio of RAC and BSA was calculated using the Beer-Lambert Law as follows (Feng et al., 2009): conjugate molar ratio= $(\varepsilon_{\text{con, 278 nm}}-\varepsilon_{\text{BSA, 278 nm}})/(\varepsilon_{\text{RAC, 274 nm}})$, where $\varepsilon_{\text{con, 278 nm}}$ is the molar extinction coefficient of conjugate at 278 nm (L/(mol·cm)), $\varepsilon_{\text{BSA, 278 nm}}$ is the molar extinction coefficient of BSA at 278 nm (L/(mol·cm)), and $\varepsilon_{\text{RAC, 274 nm}}$ is the molar extinction coefficient of RAC at 274 nm (L/(mol·cm)).

2.4 Preparation of mAb and AuNP conjugation

The mAb against RAC was obtained by immunizing a mouse with RAC–BSA as described previously (Buakeaw et al., 2016). The mAb was purified using protein G affinity chromatography. Subsequently, the AuNP label was conjugated to the mAb following the previously reported method with slight modification (Preechakasedkit et al., 2012). First, the optimal concentration of the mAb conjugated to the AuNPs was selected by mixing the AuNPs (pH 8.0) with a particle size of 20 nm with the various concentrations of the mAb (between 0 and 200 g/mL) for 30 min. Next, 0.10 g/mL NaCl was added to the mixture and the optimal condition was selected by color change visualization and UV-vis characterization. After obtaining the appropriate concentration of the mAb, the mAb–AuNP conjugate was prepared by simply mixing the AuNPs and mAb because the AuNPs and the mAb can directly conjugate via physical adsorption. The mixture was incubated under

continuous stirring at room temperature for 1 h. Subsequently, 0.03 g/mL BSA was added and incubated under continuous stirring at room temperature for 1 h to block any unbounded surface, and the mixture was centrifuged at 15 000 r/min for 30 min. Finally, the soft sediment was washed and re-suspended in 0.01 mol/L PBS (pH 7.4) containing 0.03 g/mL BSA and 0.02 g/mL sucrose and stored at 4 °C. The solution of the mAb–AuNP conjugate was characterized by a UV-vis spectrophotometer.

2.5 Preparation of the lateral flow strip test

The lateral flow strip test comprised four pads (sample, conjugate, analytical and absorbent pads). The sample and absorbent pads were used without any pre-treatment. The mAb–AuNP conjugate was dispensed on the conjugate pad using an IsoFlo Flatbed Dispenser (Imagine Technology, USA) at a flow rate of 5 μL/cm. The analytical pad contained a test line at the bottom and a control line at the top of the NCM. RAC–BSA and GAM were respectively dispensed as the test and control lines on the NCM at a flow rate of 1 μL/cm. The concentration of RAC–BSA on the test line was tested between 0.50 and 1.25 mg/mL to select an appropriate amount of RAC–BSA. After dispensing, the NCM was blocked by 0.10 g/mL BSA in 0.01 mol/L PBS (pH 7.4) and dried at 37 °C for 1 h. Finally, all the pads were attached on a plastic backing card and a cassette to be ready for use. The schematic illustration of the strip test is shown in Fig. 1a.

2.6 Assay sensitivity and cross reactivity

The strip test was used to detect RAC by applying 100 μL samples of various concentrations of standard RAC (0–1 ng/mL) to the sample pad. The cross reactivity of the strip test was also tested by loading the related compounds in the β-agonist group to the sample pad. After loading the solution, each strip test was allowed to react completely and the color intensity was evaluated by the naked eye within 5 min. RAC in the sample (as a positive control) can bind to the mAb–AuNPs leading to the non-binding between the mAb–AuNPs and immobilized RAC–BSA; thus, only one control line appeared on the NCM (Fig. 1b). In the absence of RAC (as a negative control), the mAb–AuNPs could bind with the immobilized RAC–BSA and two colored lines appeared on the NCM (Fig. 1c). For qualitative analysis assessed by the naked eye, the limit of detection (LOD) was obtained from the minimal concentration of RAC leading to the complete disappearance of color intensity at the test line. Furthermore, the color intensity was captured using an iPhone 6 plus (Apple, CA, USA) under a light-controllable box. The intensity (gray scale value) of each result was measured by ImageJ software (NIH, MD, USA) for semi-quantitative analysis, and LOD ($3SD_{blank}$/slope, where SD is standard deviation) and limit of quantitation (LOQ=$10SD_{blank}$/slope) were calculated from a semi-quantitative calibration curve.

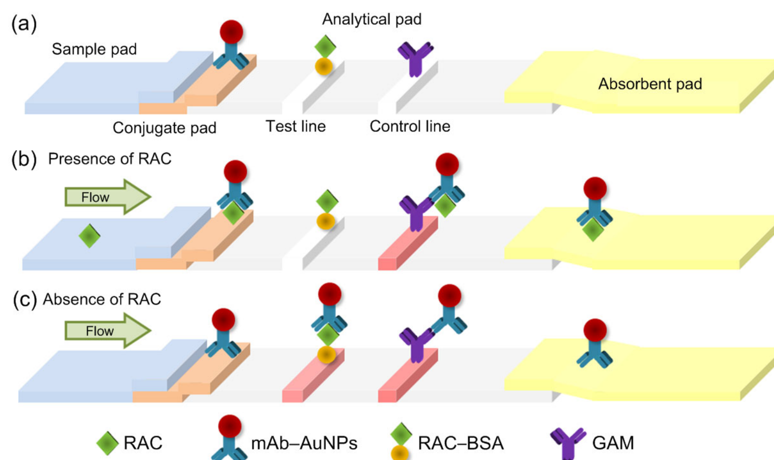

Fig. 1 RAC detection by the lateral flow strip test
(a) Schematic illustration of the lateral flow strip test; (b) Presence of RAC; (c) Absence of RAC

2.7 Sample preparation

A brand of animal feed for swine without feed additive that was purchased from the local markets in Thailand was used for sample analysis. All samples were prepared using the spiked method following a previous report with slight modification (Lin et al., 2012). First, 1 g of animal feed was weighted in a centrifuge tube, and various amounts of RAC (0–1 ng) were spiked into it. Next, 5 mL of the optimal ratio of methanol and a running buffer was added as extraction buffer and stirred for 15 min. The mixture was then centrifuged at 4000 r/min for 10 min. Subsequently, the supernatant was collected and analyzed according to the assay procedure. In addition, ELISA was also used to determine the amount of RAC in the animal feed in parallel with the result obtained from the strip test to confirm the accuracy and acceptability of this novel method.

3 Results and discussion

3.1 Conjugation of RAC and protein carrier

Due to the small molecules of RAC, a competitive format, which is the competition between RAC in the sample and an immobilized RAC to bind with an antibody, is normally used for the RAC immunoassay. The conjugation method used to prepare the RAC–protein carrier (to be used as the immobilized RAC) is an essential requirement. Therefore, the conjugation of RAC and BSA based on the Mannich reaction is reported in this study. The mechanism of the RAC–BSA conjugation is shown in Fig. 2a. The Mannich reaction involves the condensation of the aldehyde group (which can bind to the active hydrogen of RAC at the ortho position via the electrophilic aromatic substation) of RAC with the amine group of BSA to form the RAC–BSA conjugation. The conjugation via the Mannich reaction displays stable covalent bonds (Hermanson, 2008). The obtained RAC–BSA also exhibited a clear solution without any pellets. Furthermore, the UV-vis spectra, as shown in Fig. 2b, demonstrated the maximum absorbance of RAC, BSA, and RAC–BSA at 274, 278, and 278 nm, respectively. The conjugate molar ratio of RAC and BSA was calculated from the Beer-Lambert Law and was found to be 9:1 (RAC:BSA), indicating successful coupling between RAC and BSA. To accurately confirm the conjugation, the MWs of BSA and RAC–BSA were also analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) as shown in Fig. S1. From MALDI-TOF-MS data, the MW of RAC–BSA (66 691 g/mol; Fig. S1b) increased from the MW of BSA (66 090 g/mol; Fig. S1a), demonstrating the conjugation between RAC and BSA. When compared with previous methods used for conjugating RAC and BSA (Wang et al., 2015; Buakeaw et al., 2016), the conjugate molar ratios of the various methods were reported as 7:1 and 8:1. Unfortunately, the drawbacks of the previous conjugation methods are that they required complicated steps, many reagents, strong organic solvents and nitrogen gas for solvent evaporation. Therefore, the RAC–BSA conjugation following the Mannich reaction could be adopted as the conjugation method exhibiting an easy, one-step and rapid conjugation with the use of fewer reagents and no requirement for the use of organic solvents.

After the RAC–BSA conjugation, the binding between RAC, RAC–BSA and mAb was preliminarily tested by the competitive conventional ELISA (data not shown), and the results indicated that the competition between the RAC–BSA and RAC to bind with the mAb occurred as expected. After the confirmation of the binding between RAC, RAC–BSA and mAb by ELISA, the RAC–BSA and mAb were applied to fabricate the lateral flow strip test. After loading the running buffer as a negative control on the strip test, the color intensities appeared on both the test and control lines (Fig. 2c). Furthermore, the strip test with the RAC–BSA prepared via the Mannich reaction (RAC–BSA$^{(1)}$) was compared to the strip test with the RAC–BSA prepared by the previous method (Wang et al., 2015; Buakeaw et al., 2016) (RAC–BSA$^{(2)}$) using the same antibody and concentration of the RAC–BSAs. After loading the running buffer, the color intensity appeared on the test line immobilized by RAC–BSA$^{(1)}$ (Fig. S2a), and there was no color intensity on the test line immobilized by RAC–BSA$^{(2)}$ (Fig. S2b). Therefore, the RAC–BSA conjugation via the Mannich reaction could be accepted and applied to the competitive lateral flow strip test.

3.2 Optimization of the lateral flow strip test

For the competitive lateral flow strip test, the AuNPs (as label) provide visual qualitative assay as

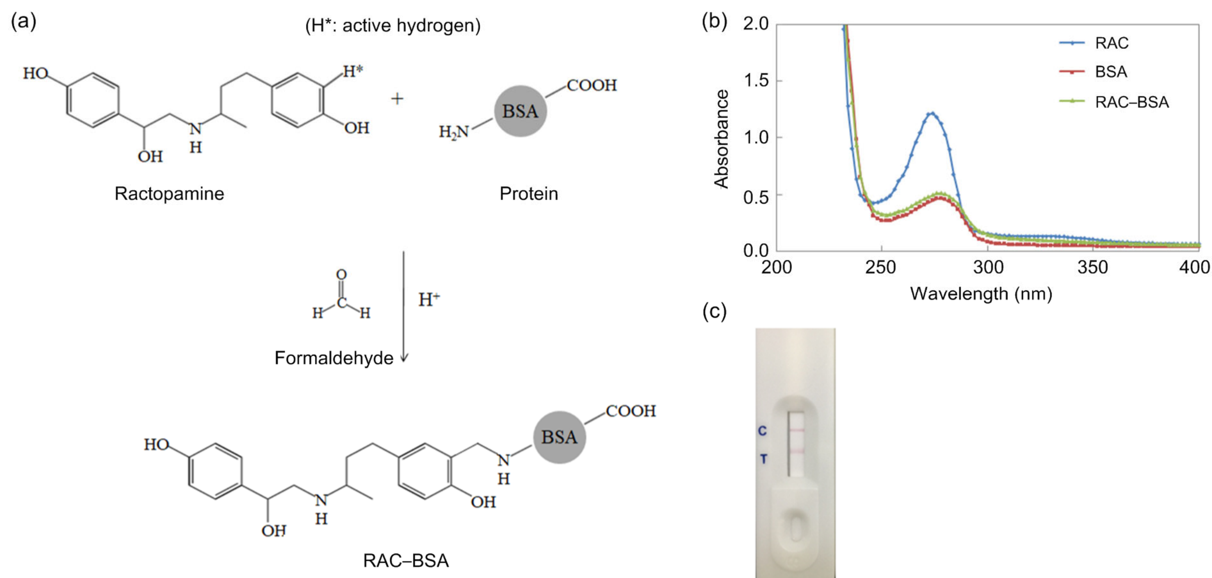

Fig. 2 Conjugate of ractopamine and bovine serum albumin (RAC–BSA)
(a) The RAC–BSA conjugation via the Mannich reaction performed using MES buffer (pH 4.7); (b) The UV-vis spectra of 0.5 mg/mL of RAC (blue line), 3.0 mg/mL of BSA (red line), and 2.0 mg/mL of RAC–BSA (green line); (c) The lateral flow strip test for RAC detection after loading 100 μL of running buffer as negative control (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

yes/no responses depending on the concentrations of RAC. Therefore, the optimal conditions for the competitive lateral flow strip test for RAC, including the effect of the concentration of mAb conjugated to AuNPs and the concentration of RAC–BSA at the test line, should be studied, similar to the checkerboard titration in the competitive ELISA (Dai et al., 2015).

3.2.1 Effect of concentration of mAb on the conjugation with AuNPs

The concentrations of the mAb (between 0 and 200 μg/mL) were studied for the selection of the minimal concentration of the mAb that was suitable for conjugation to the AuNPs. The particle size of the AuNPs was approximately 20 nm and uniform in size confirmed by a transmission electron microscopy (TEM) image (Fig. S3). The optimal concentration was selected by the color change visualization and UV-vis characterization. In case of an inappropriate amount of the mAb, the addition of NaCl after mixing mAb and AuNPs led to the aggregation of AuNPs and the color of the solution changed from red to purple. Our results indicated that the color of the solution did not change at a concentration of 100 μg/mL and the absorbance at 520 nm was initially stable, as shown in Fig. 3a. Therefore, we chose the concentration of 100 μg/mL as the optimal value for conjugating with the AuNPs. Furthermore, the conjugation between the mAb and AuNPs was also confirmed by a red shift of the peak of the conjugate (524 nm) compared with that of the free AuNPs (520 nm) (Fig. S4).

3.2.2 Effect of concentration of RAC–BSA on the immobilization at test line

The detection of RAC using the competitive format involves the competition between RAC in the samples and the immobilized RAC–BSA for the same number of antibody binding sites. The sensitivity of the strip test should be visually assessed, starting from the minimal concentration of RAC to the concentration at which there is complete disappearance of the color intensity at the test line. The concentration of the immobilized RAC–BSA was therefore important for the preparation of the strip test because over concentration of RAC–BSA causes low sensitivity. The RAC–BSA concentrations of 0.50, 0.75, 1.00, and 1.25 mg/mL were studied and the running buffer was used as the negative control for evaluating the optimal value. The results observed by the naked eye as shown in Fig. 3b indicated that the color intensities of the test line were constant at the concentrations between 0.75 and 1.25 mg/mL but decreased at

0.50 mg/mL of RAC–BSA. Therefore, 0.75 mg/mL of RAC–BSA, which was the minimal concentration that gave the maximum color intensity for the negative control on the test line, was selected as the optimal concentration to be immobilized on the test line.

3.3 Assay sensitivity, cross reactivity, and stability

Under optimal conditions, the running buffer and standard RAC, together making up 100 μL, were loaded on the sample pad. The ability to detect standard RAC was evaluated at concentrations between 0 and 1 ng/mL. In the case of the competitive format for qualitative analysis assessed by the naked eye, the highest color intensity appeared in the absence of RAC (negative control) and the color intensity decreased with increasing concentrations of RAC. LOD, as viewed by the naked eye, was defined as the minimal amount of standard RAC resulting in no color intensity at the test line. As shown in Fig. 4, the color intensity of the test line completely disappeared at 0.75 ng/mL of RAC, whereas that of both the test and control lines appeared at concentrations lower than 0.75 ng/mL. Therefore, the LOD for the standard RAC was fixed at 0.75 ng/mL. In the previous reports that used the AuNP strip test for standard RAC detection, LODs, as viewed by the naked eye, were 2 and 3 ng/mL (Li et al., 2010; Gu et al., 2016), demonstrating that our strip test has the highest sensitivity for RAC and therefore should be practically applied for the detection of RAC in real samples.

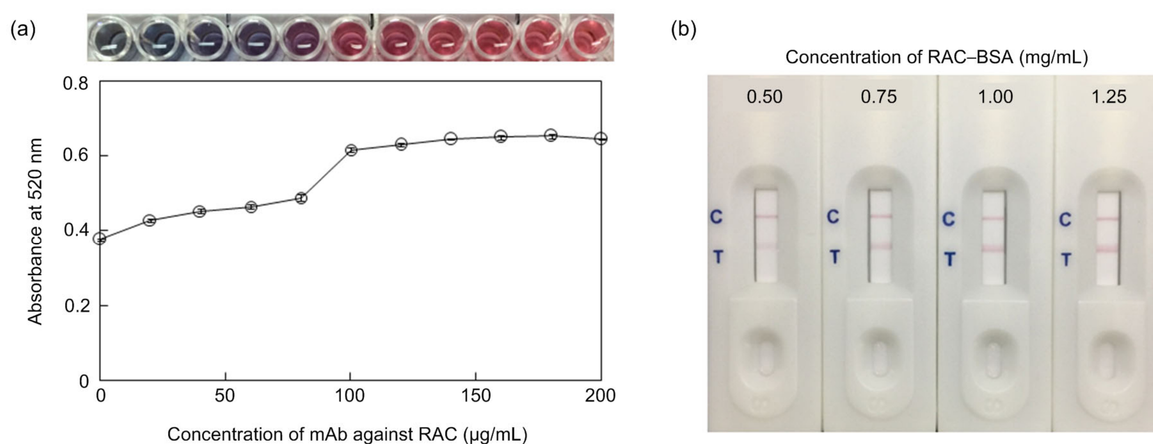

Fig. 3 Optimization of the lateral flow strip test

(a) The effect of concentrations of mAb between 0 and 200 μg/mL on the conjugation with AuNPs characterized by the visualization of color change and UV-vis spectrophotometer. The data are expressed as mean±SD (n=3). (b) The effect of concentrations of RAC–BSA (0.50, 0.75, 1.00, and 1.25 mg/mL) for the immobilization at test line (n=3)

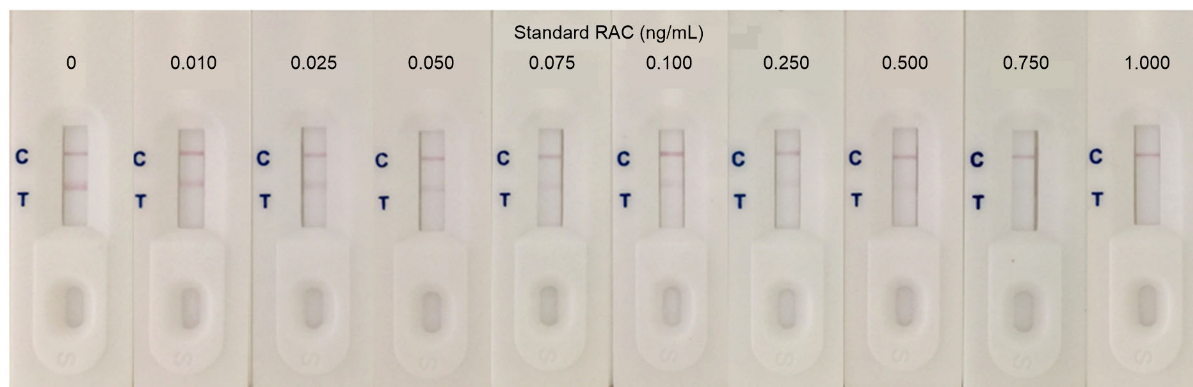

Fig. 4 Lateral flow strip test of standard RAC at concentrations between 0 and 1.000 ng/mL

For the cross-reactivity test, high concentrations of the related compounds were applied to the strip test for RAC detection. The related compounds included salbutamol (SAL), clenbuterol (CLB), terbutaline (TER), norepinephrine (NE), and phenylephrine (PHE) at a concentration of 1000 ng/mL and were studied in this work along with the negative (the absence of RAC) and positive (the presence of RAC) controls. The results shown in Fig. S5 indicated that the test line disappeared only when RAC was present, whereas it appeared on the strip test loaded with SAL, CLB, TER, NE, PHE, and in the negative control (no RAC). Therefore, the developed strip test demonstrated a high specificity for RAC detection.

Furthermore, the storage stability was studied after keeping the strip test at room temperature for 0 to 5 months. After loading a negative control (Fig. S6a), two color lines appeared on all the strip tests while the test line still disappeared on all strip tests in the presence of 1 ng/mL of RAC (Fig. S6b). These results indicated the long-term stability of the strip test (more than 5 months).

3.4 Sample application

Animal feed was selected for the application of the strip test because we found no report for the use of the strip test for the direct detection of RAC in animal feed. For the extraction of animal feed, an organic solvent, particularly methanol, is normally required. However, there is a report indicating that an increase in the methanol concentration led to a decrease in the sensitivity of the conventional ELISA (Gu et al., 2016) in detecting RAC. Due to the effect of methanol, the volume ratios of methanol and running buffer (together used as the extraction buffer) were studied at 0:100, 5:95, 10:90, 20:80, and 40:60. We found that the color intensities of the test and control lines remained constant up to the ratio of 10:90 and then decreased (Fig. S7). The decrease in the intensity at high concentrations of methanol resulted from the interference of the matrix effect and the color of animal feed. Therefore, the volume ratio of 10:90 (methanol: running buffer) was used for the extraction buffer.

After optimizing for the extraction buffer, we measured RAC in animal feed (either spiked with RAC at final concentrations of 0.075–1.000 ng/g or not spiked with RAC) using the strip test. As shown in Fig. 5a, the color intensity of test line clearly disappeared at 1 ng/g of the RAC-spiked sample. LOD, as viewed by the naked eye, was thus found to be 1 ng/g of the spiked RAC in animal feed. For the application of the strip test in animal feed, the LOD of the spiked RAC (1 ng/g) was slightly higher than the LOD of the standard RAC (0.75 ng/mL) because the detection system could be interfered by the matrix effect due to unknown substances present in animal feed. However, the LOD of the spiked RAC in this work is still lower than the amount of RAC in the feed supplement (5 μg/g) (Patience et al., 2009), the MRLs in edible tissues (10–90 ng/g) and the previous reports on the use of AuNP strip test in swine urine (>2 ng/mL) (Li et al., 2010; Gu et al., 2016), indicating that the proposed strip test is acceptable for the detection of RAC in sample. To enhance the capability of the normal strip test that evaluates only the yes/no response, a semi-quantitative analysis could be easily developed using ImageJ software. The relationship between the change in Δgray intensity and the concentrations of the non-spiked and spiked RAC in animal feed at 0.075–1.000 ng/g, as shown in Fig. S8, displayed a negative sigmoidal-shaped curve that is similar in principle to the competitive format (Hage et al., 1993). A semi-quantitative calibration curve was then constructed by plotting the Δgray intensity and the logarithm of the concentrations of the spiked RAC, as shown in Fig. 5b. A linear range was observed between 0.075 and 0.750 ng/g, with a good correlation of 0.992 (n=6). The calculated LOD and LOQ were found to be 0.10 and 0.33 ng/g, respectively. The smaller SDs observed based on the independent strip tests indicate high reproducibility.

To test the practical applicability of the strip test, RAC spiked into animal feed at 0.10, 0.25, and 0.50 ng/g was measured to calculate for percent recovery and relative standard derivation (RSD) percentage using an independent strip test on the same day (intra-assay) and on three different days (inter-assay), and the values obtained were compared with those obtained using the conventional method (ELISA) as summarized in Table 1. The intra-assay (n=6) recovery percentage was in the range of 96.9–100.2 with RSD of 2.5%–2.8%, and the inter-assay (n=3) recovery percentage was 96.4–103.7 with an RSD of 3.1%–3.6%. The results demonstrated smaller RSD percentages for both intra- and inter-assays, demonstrating the precision of the developed method.

Table 1 Comparison of lateral flow strip test and conventional method (ELISA) for RAC detection in animal feed

Spiked RAC (ng/g)	RAC by ELISA (n=6) (ng/g)	Lateral flow strip test					
		Intra-assay (n=6)			Inter-assay (n=3)		
		RAC (ng/g)	Recovery (%)	RSD (%)	RAC (ng/g)	Recovery (%)	RSD (%)
0.10	0.09±0.01	0.09±0.01	96.9	2.7	0.11±0.01	103.7	3.6
0.25	0.24±0.02	0.24±0.02	98.3	2.8	0.22±0.03	96.4	3.1
0.50	0.52±0.04	0.51±0.02	100.2	2.5	0.53±0.02	100.8	3.3

The values of RAC concentrations are expressed as mean±SD

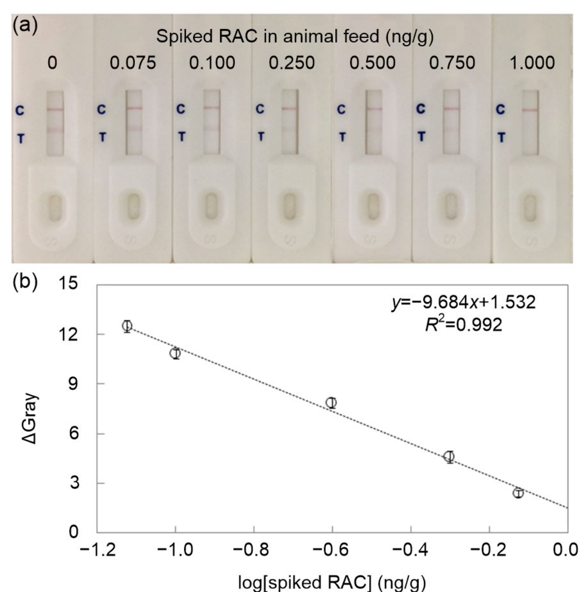

Fig. 5 Application of the lateral flow strip test for RAC detection in animal feed
(a) The detection of the non-spiked and spiked RACs in animal feed at 0.075–1.000 ng/g; (b) The calibration curve between the Δgray intensity and the logarithm of the concentrations of the spiked RAC at 0.075–0.750 ng/g. The data are expressed as mean±SD (n=6)

To compare the results obtained using the strip test with those obtained using ELISA, a paired t-test at a 95% confidence interval was used for animal feed analysis. The calculated t-value obtained by the strip test was lower than the critical t-value, indicating no significant difference between the strip test and ELISA. Therefore, the proposed strip test, with high reproducibility and precision, could have strong potential as an alternative device for the early screening of RAC in animal feeds.

4 Conclusions

We successfully developed a novel method for conjugating RAC and BSA based on the Mannich reaction, providing advantages over the previous conjugation methods in terms of simplicity, rapidity, single step, and the requirement of fewer reagents. This conjugation method gave RAC–BSA with a reasonable mole coupling ratio of 9:1. The RAC–BSA conjugation was applied to preparing the lateral flow strip test for RAC detection in animal feed. The LOD was found using two methods, including the qualitative analysis through naked eye visualization and semi-quantitative analysis through the use of the semi-quantitative calibration curve. The LOD for RAC in animal feed by the naked eye was found to be 1 ng/g (detected within 5 min) without cross reactivity with the related compounds. The semi-quantitative analysis for RAC in animal feed using the strip test displayed linearity in the range of 0.075–0.750 ng/g with a good correlation of 0.992. The calculated LOD and LOQ were found to be as low as 0.10 and 0.33 ng/g of RAC, respectively. For the practical applicability of the test, the percent recovery could be observed in the range of 96.4%–103.7% with an RSD of 2.5%–3.6% for intra- and inter-assays, and the results obtained using the strip test agreed well with those obtained using ELISA. Therefore, the construction of a one-step, low-cost, and portable strip test was successfully applied for the highly sensitive and specific detection of RAC with reproducibility and precision. Furthermore, the conjugation method based on the Mannich reaction could be further recommended to conjugate various compounds containing active hydrogen with a protein carrier, and the proposed strip test could be applied for the detection of RAC in other samples, such as swine urine and meats.

Contributors

Pattarachaya PREECHAKASEDKIT performed the experiment research and data analysis, wrote and edited the manuscript. Nattaya NGAMROJANAVANICH performed data analysis and edited manuscript. Nanthika KHONGCHAREONPORN and Orawon CHAILAPAKUL contributed to the study design, experiment performing, data analysis, and manuscript

writing and editing. All authors read and approved the final manuscript.

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Compliance with ethics guidelines

Pattarachaya PREECHAKASEDKIT, Nattaya NGAMROJANAVANICH, Nanthika KHONGCHAREONPORN, and Orawon CHAILAPAKUL declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Results of MALDI-TOF-MS analysis

Fig. S2 Lateral flow strip test for RAC detection using the RAC–BSA$^{(1)}$ prepared via the Mannich reaction and the RAC–BSA$^{(2)}$ prepared using previous method for the immobilization on test line

Fig. S3 TEM image of AuNPs

Fig. S4 UV-vis spectra of AuNPs and monoclonal antibody against RAC–AuNPs conjugated

Fig. S5 Cross reactivity test by loading running buffer, RAC, SAL, CLB, TER, NE, and PHE

Fig. S6 Storage stability after keeping the strip tests for 0 to

5 months after loading running buffer as negative control and 1 ng/mL of RAC as positive control

Fig. S7 Effect of the volume ratio of methanol and running buffer of 0:100, 5:95, 10:90, 20:80, and 40:60 toward the lateral flow strip test

Fig. S8 Relationship between the Δgray intensity and the concentrations of the non-spiked (0) and spiked RAC in animal feed at 0.075–1.000 ng/g

中文概要

题　目：一种新型莱克多巴胺-蛋白质载体结合技术在侧向流试纸条检测动物饲料中莱克多巴胺含量中的应用

目　的：开发一种新型简单快速的克莱多巴胺-蛋白质载体结合方法，用于侧向流试纸条检测动物饲料。

创新点：基于曼尼希反应的克莱多巴胺-牛血清蛋白（RAC–BSA）结合方法，具有一步性、简单、快速，且所需试剂少的优点。

方　法：通过曼尼希反应产生 RAC–BSA 结合物，制备竞争型侧向流试纸条。将 RAC 标记于猪饲料中，以体积比 10:90 的甲醇:电泳缓冲液作为提取缓冲液，在 25 min 内完成样品制备。通过裸眼观察定性评估 RAC–BSA 结合物的免疫应答以及检测灵敏性，通过智能手机和电脑软件（ImageJ）进行半定量分析。

结　论：在最佳条件下，侧向流试纸条检测的检出限（LOD）在 5 min 裸眼评估下为 1 ng/g；半定量分析中，其线性范围为 0.075~0.750 ng/g，计算的 LOD 为 0.10 ng/g，定量限为 0.33 ng/g，且相关性较好（R^2=0.992)。回收率为 96.4%~103.7%，同实验内及不同实验间的相对标准偏差为 2.5%~3.6%。通过与酶联免疫吸附法对比，该侧向流试纸条检测结果具有较高的准确性和特异性。因此，通过曼尼希反应制备 RAC–BSA 结合物是一步的简单的结合法，可用于侧向流试纸条检测法。

关键词：克莱多巴胺；克莱多巴胺-牛血清蛋白；曼尼希反应；侧向流检测；饲料添加剂