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ARTICLES

Development of a Chemiluminescent ELISA for Determining Chloramphenicol in Chicken Muscle

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An indirect competitive enzyme-linked immunosorbent assay (ELISA) with chemiluminescent (CL) detection for chloramphenicol (CAP) in chicken muscle was developed. CAP-specific polyclonal antibody was raised in rabbit with a CAP-succinate derivative conjugated with bovine serum albumin. Luminol solution was used as the substrate of horseradish peroxidase. The detection limit was 6 ng/L. The CL-ELISA was 10 times more sensitive compared to the colorimetric-ELISA. When CAP was spiked in chicken muscle at levels of 0.05−5 μg/kg, recoveries ranged from 97 to 118% with coefficients of variation of 6−22%. In an actual residue study, the results obtained by CL-ELISA correlated well with those obtained by gas chromatography with microcell electron capture detector. The residue levels of CAP in treated chicken decreased with time and dropped rapidly after the first 6 h from around 50 to 10 μg/kg. After 3 days, CAP was not detected in chicken muscle. The developed method is therefore suitable for screening of CAP in chicken muscle samples.

KEYWORDS: Chemiluminescent ELISA; chloramphenicol; chicken muscle

INTRODUCTION

Chloramphenicol (CAP) is an effective broad spectrum antibiotic widely used in veterinary practices. Due to its side effects in humans, especially fatal bone marrow depression and aplastic anemia, the use of CAP for treatment of food-producing animals is prohibited in many countries including China. Residues of CAP in the human food supply are proclaimed to be undetectable. However, illegal use may lead to potential exposure of CAP to consumers. The detection limit of the analytical methods is crucial and decisive to control CAP residues at trace levels. Therefore, it is necessary to develop sensitive methods for determining CAP residues in animal tissues.

A variety of methods such as LC (*1*, *2*), GC (*3*−*6*), GC-MS (*7*−*9*), LC-MS (*10*−*12*), and ELISA (*13*−*15*) have been reported for the determination of CAP residues in animal tissues. However, most methods could not reach a detection limit of 0.1 μg/kg, which is required for monitoring CAP residues in animal tissues. Chemiluminescent ELISA (CL-ELISA) is a good alternative method for screening samples. The enhanced chemiluminescent (ECL) reaction offers the possibility of improving the sensitivity of the immunoassays by at least 2−3 orders of magnitude compared to conventional colorimetric detection. This advantage of CL-ELISA makes it a useful detection system for residue analysis. Up to now, several chemiluminescent methods have been established for residue analysis of veterinary drugs and environmental contaminates (*16*−*26*).

The purpose of this study was to develop a sensitive chemiluminescent ELISA for the determination of CAP in chicken muscle. The developed method was compared with the conventional colorimetric ELISA and applied to the analysis of incurred muscle samples from CAP-treated chicken.

MATERIALS AND METHODS

Materials. CAP, CAP-succinate, goat anti-rabbit IgG (whole molecular conjugated to horseradish peroxidase, GaRIgG−HRP), ovalbumin (OVA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). The CL substrate solution called SuperSignal was purchased from Pirece (Rockford, IL). Other reagents were of analytical quality and supplied by Beijing Reagent Corp. (Beijing, China). All aqueous solutions and buffers were prepared with water purified on a Milli-Q system (Millipore, Bedford, MA). The following buffers were used: (A) coating buffer, 0.05 M carbonate/bicarbonate buffer, pH 9.6; (B) blocking buffer, 0.01 M sodium phosphate-buffered saline (PBS) with 0.5% casein, pH 7.4; (C) assay

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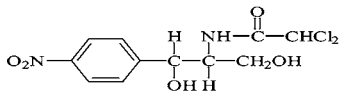
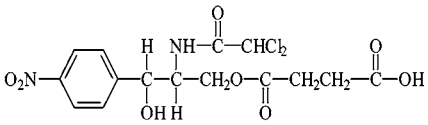
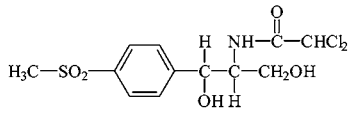
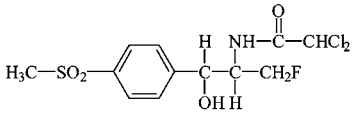
Table 1. Specificity of PAb to CAP and Its Analogues

Compound	Structure	I_{50} (μg/L)	Cross-reactivity (%)
CAP		1.3	100
CAP succinate		0.2	722
thiamphenicol		957	0.1
florfenicol		1430	0.1

buffer, PBS with 0.01% Tween-20 (PBST); and (D) 0.2 M sodium phosphate, pH 7.2 (PB).

Apparatus. Chemiluminescence was measured with a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). The colorimetric-ELISA was made by a Sunrise microtiterplate reader (TECAN, Groedig, Austria). The transparent 96-well microtiter plates (Nalge Nunc, Hereford, U.K.) were used for the colorimetric assay and the black polystyrene microtiter wells (Costar, Ithaca, NY) for chemiluminescent detection.

Production of Polyclonal Antibody. Protein−CAP conjugates were synthesized by the activated ester method, using the procedure previously reported (*13*). Briefly, 700 mg (1.6 mmol) of CAP-succinate was dissolved in 50 mL of distilled water. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (635 mg, 1.5 μmol) in 10 mL of distilled water and 175 mg (1.5 μmol) of *N*-hydroxysuccinimide in 10 mL of distilled water were added (pH 5.3). The reaction mixture was incubated with stirring for 1 h at room temperature. Activated CAP-succinate (630 μmol) was added to 200 mg (3 μmol) of BSA in 20 mL of PB. The reaction mixture was incubated with stirring for 2 h at room temperature followed by overnight incubation at 4 °C. The reaction mixture was dialyzed against 0.01 mmol/L PB for 5 days. The dialyzed solution was characterized by UV−vis spectrophotometry. CAP−BSA conjugate was used as immunogen, and CAP−OVA conjugate was used as coating antigen. The immunogen was injected subcutaneously in three New Zealand White rabbits. After five booster injections, the sera were collected and purified by saturated $(NH_4)_2SO_4$. The PAb was used further in the study. An indirect competitive ELISA method was developed and used to characterize the PAb. The ELISA procedure was similar to the previously reported method (*27*).

ELISA Procedure. The wells of microtiter plates were coated with 100 μL of CAP−OVA solution dissolved in buffer A (1.7 μg/mL) overnight at 4 °C. The wells were washed with PBST three times and blocked with 200 μL/well of buffer B, and the plates were incubated at 37 °C for 1 h. After the plates had been washed five times with PBST, 80 μL/well of CAP standard and 20 μL/well of PAb at a dilution of 1/10000 in buffer C were added, respectively. Following the incubation and washing procedure, 100 μL/well of GaRIgG−HRP (1:2000 dilution in PBST) was added with incubation at 37 °C for 1 h. The plates were then washed. Chemiluminescence was measured immediately after the addition of 100 μL of SuperSignal substrate solution.

Optimization of Chemiluminescent ELISA. Several physicochemical factors that influenced the chemiluminescent ELISA performance were studied. Modifications of RLU_{max} (RLU, relative light unit) and I_{50} parameters of the standard curve were evaluated under different conditions. To evaluate the influence of Tween-20, buffer ionic strength, and buffer pH, standard curves were prepared in purified water, whereas PAbs were prepared as follows: (1) PAbs were added to serial dilutions of Tween-20 (from 1 to 0%, v/v) in PBS; (2) a constant concentration of PAb was added to serial dilutions (from 10× to 0.25× PBS) of a concentrated buffer; (3) a constant concentration of PAb in 1× PBS at the different pH values (from 5.0 to 9.0).

Standards were run in quadruplicate wells, and mean chemiluminescence intensity values were processed. Standard curves were obtained by plotting chemiluminescence intensity against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using Origin (version 7.5, Microcal, Studio City, CA)

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the asymptotic maxmum (chemiluminescence intensity in the absence of analyte, RLU_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentratin that reduces RLU_{max} to 50%), and D is the asymptotic minimum (background signal).

Sample Preparation. A 5 g chicken muscle sample was homogenized and mixed with 15 mL of acetonitrile/water (84:16, v/v). After vortexing for 1 min, the mixture was centrifuged for 5 min at 5000*g*. Then 3 mL of supernatant was mixed with 1 mL of 0.5 M NaCl solution in a centrifuge tube; 3 mL of acetic ester was added, and the mixture was shaken for 1 min. The upper fraction was separated and evaporated to dryness. The residue was dissolved in 1 mL of buffer D and 1 mL of hexane. After centrifugation for 5 min at 5000*g*, the lower fraction was transferred and diluted 10-fold, and then 80 μL of the solution was added to the black polystyrene microtiter wells and measured.

CAP Residue Study. Eighty 8-week-old broilers (AA species, Zhengda Co., Beijing, China) were randomly divided into six groups and raised in brooders provided with fresh feed and water every day. The control group was given drug-free feed; five groups were given feed treated with 800 mg/kg CAP (w/w). The broilers were fed for 5 consecutive days. One group was slaughtered after 0 h, 6 h, 24 h, 2 day, and 3 day withdrawal periods, respectively. The muscle tissue samples were collected and frozen at −20 °C until analysis by the CL-

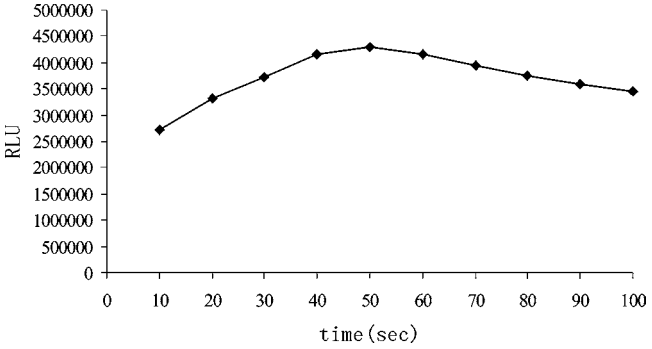


Figure 1. Kinetic profile of the light emission obtained using SuperSignal as the HRP substrate at B_0 experimental conditions.

ELISA and gas chromatography with microcell electron capture detection (GC-μECD) methods.

Analysis of Chicken Muscle Samples by GC-μECD. Sample extraction and cleanup procedures used were an adaptation of previously reported procedures (*6*). An Agilent model 6890 gas chromatograph equipped with a capillary column (HP-5, 5% phenyl methyl silicone, 30.0 m × 320 μm × 0.50 μm nominal, model 19091J-113) and a ^{63}Ni electron capture detector (Agilent Technologies, Palo Alto, CA) was used. Samples (3 μL) were injected splitless. Nitrogen was used as carrier gas. The following temperature program was applied: start from 150 °C (hold time of 0.5 min), then ramp at 30 °C/min to 270 °C, holding for 5 min. The detector was operated at 300 °C.

RESULTS AND DISCUSSION

PAb Preparation and Characterization. The sera from the rabbits showed high titer (1:640000) in the ELISA. The sera were characterized using an indirect competitive ELISA method. The specificity of the PAbs was evaluated using three compounds structurally related to CAP. The PAbs showed insignificant cross-reactivity with other fenicol drugs, such as thiamphenicol and florfenicol. Other structurally unrelated drugs were also tested, including clenbuterol, ractopamine, sulfamethazine, metronidazole, and furazolidone. No cross-reactivity was observed. Therefore, the PAb was suitable for establishing immunoassays for screening CAP in animal tissues. The selectivity of CAP and CAP analogues is shown in **Table 1**.

Chemiluminescent Detection Optimization. The commonly used CL substrates for HRP are luminol and H_2O_2. A variety of substituted phenols and arylboronic acid derivatives have been applied as enhancers for this system, such as 4-iodophenol, 4-phenylboronic acid, and 4-iodophenylboronic acid (*28*). The system exhibited a glow-type light emission. One commercially available CL substrate for HRP was used in this CL-ELISA. The kinetic profile of the light emission obtained in the assay at B_0 conditions is shown in **Figure 1**. The CL values are proportional to the HRP activity. The optimized experimental conditions involve the addition of 100 μL of SuperSignal substrate to each well and measurement of light output after 1 min.

Physicochemical Parameter Optimization. Several experimental factors influencing the CL-ELISA performance were studied. The RLU_{max}/I_{50} ratio has been shown to be a useful parameter to estimate the effect of a certain factor on the ELISA performance. The highest ratio indicates the highest sensitivity (*29*). Because surfactants (such as Tween-20) are commonly used in the ELISA to reduce nonspecific interactions, their influence on assay performance was examined. **Figure 2a** shows the variation of the RLU_{max}/I_{50} ratio as a function of the concentration of Tween-20. Higher and lower Tween-20 concentrations reduced the RLU_{max}/I_{50} ratio, so the use of Tween-20 should be limited to 0.01% in the assay.

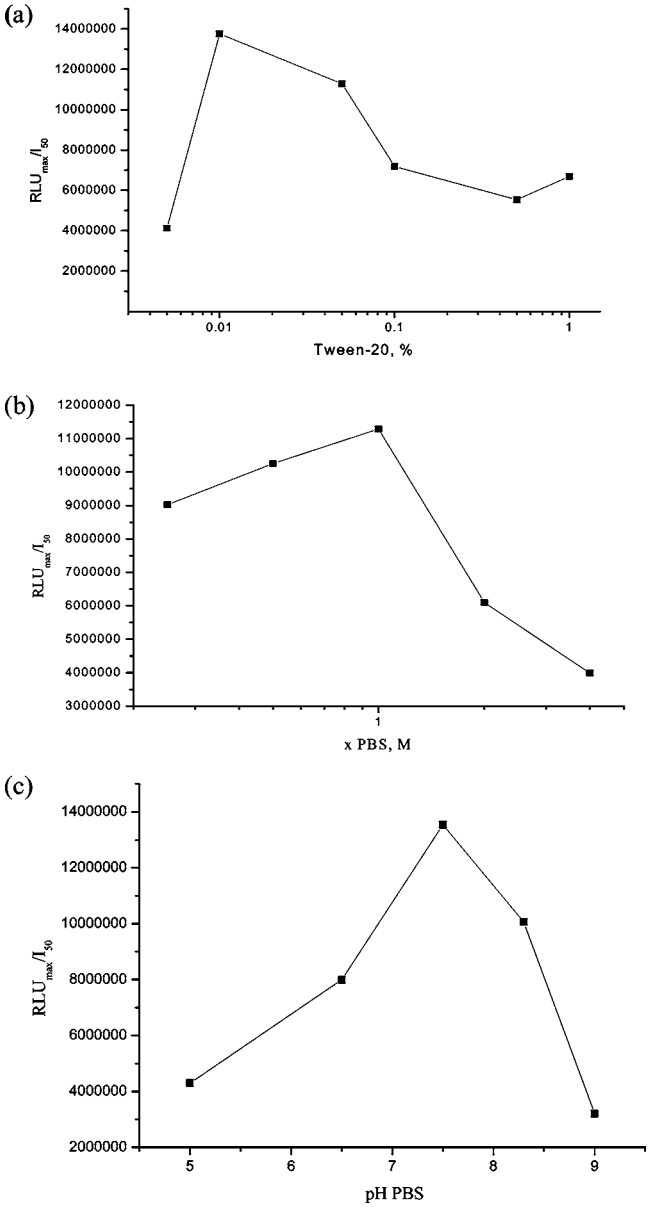


Figure 2. Influence of (**a**) Tween-20 concentration, (**b**) buffer ionic strength, and (**c**) buffer pH on the RLU_{max}/I_{50} ratio of CL-ELISA standard curve for CAP.

Because antigen−antibody binding is characterized by weak intermolecular bonds, changes in ionic strength or pH could affect this interaction. As shown in **Figure 2b**, the RLU_{max}/I_{50} ratio increased with salt concentrations up to 1× PBS. This salt concentration was selected for the dilution buffer of the antibody. **Figure 2c** shows the dependence of the RLU_{max}/I_{50} ratio on pH. This ratio reached a maximum at pH 7−8 for the assay; a pH of 7.4 was chosen as optimum.

Assay Sensitivity. The sensitivity was investigated using CAP standard. CAP calibration curves were obtained under the optimized conditions. A nonlinear equation established the best fit of the points over the entire range of the assay as shown in **Figure 3**. The linear working range determined as the concentration resulting in 20−80% inhibition of maximal chemiluminescence intensity was 0.05−12.5 μg/L. The limit of detection (LOD), determined as CAP concentration giving a 10% inhibition of the maximal chemiluminescence intensity, was 6 ng/L. The CL-ELISA was ≈10 times more sensitive compared to the colorimetric ELISA previously reported (*14*).

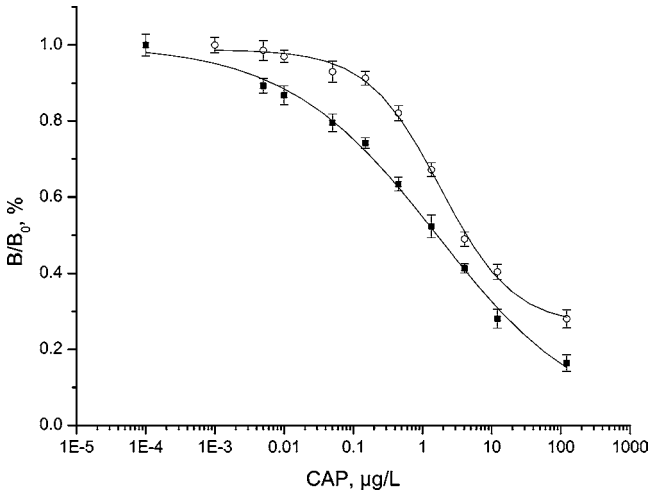


Figure 3. Normalized standard curve by CL-ELISA (■) for CAP under optimized conditions compared to the standard curve obtained by colorimetric-ELISA (○).

Table 2. Recoveries of CAP from Spiked Chicken Muscle Tissues by CL-ELISA and ELISA ($n = 5$)

	CL-ELISA			ELISA		
CAP added (μg/kg)	CAP detected, $x \pm$ SD (μg/kg)	recovery (%)	CV (%)	CAP detected, $x \pm$ SD (μg/kg)	recovery (%)	CV (%)
0.05	0.06 ± 0.01	118	22	ND[a]		
0.1	0.10 ± 0.02	101	16	ND		
0.5	0.54 ± 0.07	108	13	0.38 ± 0.11	76	30
1	0.98 ± 0.19	98	19	0.73 ± 0.09	73	12
2.5	2.58 ± 0.20	103	8	2.23 ± 0.22	89	9
5	4.86 ± 0.28	97	6	4.12 ± 0.64	82	15

[a] Not determined.

Analysis of CAP-Spiked Samples. Recovery experiments were conducted to evaluate the accuracy and precision of the CL-ELISA. The examination of sample matrix effects was performed with extracts of drug-free chicken muscle. To remove the matrix interference, the extracts were further diluted 1:1, 1:5, and 1:10. The result of the 1:10 dilution was comparable to that of the standard curve, and the result of the 1:1 dilution deviated from the standard curve. Therefore, the extracts of fortified samples were diluted 1:10 before analysis.

Five muscle samples known to be free from CAP were spiked, and known amounts of CAP were added into control chicken muscle at desired concentrations and then assayed in quadruplicate. When CAP was fortified at levels of 0.05, 0.1, 0.5, 1, 2.5, and 5 μg/kg, the recoveries of CAP ranged from 97 to 118% with coefficients of variation (CVs) of 6−22%. The samples were also analyzed by a colorimetric ELISA method. Both methods gave similar results in recovery studies at fortified levels of 0.5, 1, 2.5, and 5 μg/kg. The detection results of the accuracy and precision test are listed in **Table 2**.

Analysis of CAP in Actual Tissue Samples. To demonstrate the applicability of the chemiluminescent ELISA for the determination of CAP residues in real samples, incurred muscle samples from CAP-treated chicken were detected using the established CL-ELISA method. The results were also obtained using the colorimetric ELISA and GC-μECD methods. We found that the results of the CL-ELISA method were higher than those of the colorimetric ELISA and lower than those of the GC-μECD method as shown in **Table 3**. However, the results obtained by the CL-ELISA were highly correlated with those obtained by the colorimetric ELISA and GC-μECD methods ($r^2 = 0.98$ and 0.94, respectively), showing that the CL-ELISA can detect the presence of CAP in chicken muscles both qualitatively and quantitatively. The residue levels of CAP in chicken muscle decreased with time and dropped off rapidly after the first 6 h. After 3 days, no residues were detected by either GC-μECD or CL-ELISA. False-negative and false-positive results were not revealed during the analysis.

Table 3. Analysis of CAP-Treated Chicken Muscles ($n = 3$)

withdrawal time (h)	CAP, $x \pm$ SD (μg/kg)		
	CL-ELISA	ELISA	GC-μECD
0	49 ± 7	46 ± 9	53 ± 5
6	10 ± 3	9 ± 2	12 ± 3
24	1 ± 0.4	0.7 ± 0.3	1.1 ± 0.2
48	0.1 ± 0.03	ND	0.13 ± 0.05
72	ND	ND	ND

[a] Not determined.

In this study, we established an indirect competitive CL-ELISA based on the ECL reaction using a luminol−hydrogen peroxide system. The CL-ELISA had significantly better sensitivity and required less time than similar colorimetric ELISAs. The chemiluminescent immunoassay reported earlier used direct competitive ELISA for the determination of CAP in milk (*30*). To date, there is no report on the application of CL-ELISA for the determination of CAP residues in animal tissues. Application to the analysis of the fortified and real chicken muscle samples proved that the method described here is simple, highly efficient, and sensitive. It is suitable for screening CAP residues in animal tissues.

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