Quantitative Analysis of Chloramphenicol Residues in Shrimp Muscle Tissues by Chemiluminescent Enzyme Immunoassay

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Abstract

Chuanlai X., Cifang P., Kai H., Zhengyu J., Wukang W. (2005): **Quantitative analysis of chloramphenicol residues in shrimp muscle tissues by chemiluminescent enzyme immunoassay**. Czech J. Food Sci., **23**: 251–256.

A competitive indirect chemiluminescent enzyme immunoassay (ic-CLEIA) has been developed for the determination of chloramphenicol (CAP) residues in shrimp. After the optimisation of four physico-chemical parameters, i.e. incubation time, concentration of Tween-20, concentration of PBS and its pH, the method developed gave a limit of detection of 0.01 ng/ml and a detection range from 0.03 ng/ml to 23.7 ng/ml, with an ED_{50} of 0.47 ng/ml. The developed method has been validated on spiked shrimp samples in terms of precision (intra- and interassay coefficient variations of less than 10% and 15%, respectively), and of accuracy (mean recovery from 95% to 123%). All these parameters being better than those of the ELISA method which is widely used to detect chloramphenicol, it may be suggested that the CLEIA method can be used to detect aquatic samples instead of ELISA.

Keywords: chemiluminescent enzyme immunoassay; aquatic product; chloramphenicol; residues; food analysis

As chloramphenicol (CAP) has many side effects, e.g. it may produce severe or fatal bone marrow depression and aplastic anaemia, and a syndrome of cyanosis and cardiovascular collapse known as the "grey syndrome" may also occur, particularly in neonates, its use is banned in all the foods (Water *et al.* 1987; Winston 2002; Takino *et al.* 2003).

Various analytical methods have been reported for the determination of CAP residues in edible tissues (Nagata & Saeki 1992; Pfenning *et al.* 1998, 2000; Kolosova *et al.* 2000; Riet *et al.* 2003). They include a bio-assay method (Swab test on premises, STOP), an instrumental method (high efficient liquid chromatography with mass spectrometric detection and gas chromatography with mass spectrometric detection), and immunoassay (ELISA). All these methods have their disadvantages. The specificity and the sensitivity of the bio-assay method are low; the equipment used in the instrumental method is expensive and the operation is complicated; the operation of EIA is quick and simple, but the detection limit in the present colorimetric ELISA is only 0.1 ng/ml, which is the same as the maximal residual limit of chloramphenicol; it is actually applied at the lower limit of its dynamic range (Kim *et al.* 1995; Nagata & Oka 1996; Hirsch *et al.* 1998; Impens *et al.* 2003; Shen *et al.* 2003).

In order to improve the detection ability of CAP in aquatic product samples, we have developed a quantitative chemiluminescent enzyme immu-

noassay (CLEIA). Under the optimum conditions, the sensitivity of CLEIA can reach 0.01 ng/ml, the linear range being between 0.03–23.7 ng/ml.

MATERIAL AND METHODS

Reagents. BSA was obtained from Shanghai Baiao Company (Shanghai, China). The SuperSignal® substrate was purchased from Hyclone-Pierce (Logan, USA). Anti-CAP mouse monoclonal antibody was bought from Abcam Ltd. (Cambridge, United Kingdom). CAP-BSA was synthesised by the mixed anhydride reaction (Huang *et al.* 1991) in our laboratory.

Deionised water was purified on a Milli Q system. The buffers used were as follows: (A) The coating solution consisted of a 0.05 mol/l carbonate-bicarbonate buffer (pH 9.6); (B) The washing solution was a PBS buffer (pH 7.4) containing 0.02% (v/v) Tween-20 and (C) the assay buffer was the washing buffer containing 1% (w/v) BSA. CAP standard solution was a kind gift of the Academy of Chinese Import and Export Inspection.

Instruments. A chemiluminescence microtiter reader Luminoskan Ascent, multichannel pipette and 96-well black polystyrene microtiter plates were obtained from Thermo Labsystems (Helsinki, Finland). The horizontal whirly shaker was purchased from Changzhou guohua electronic factory (Changzhou, China).

Chemiluminescent enzyme immunoassay procedure. The CAP-BSA solution was diluted to 0.2 g/l with buffer A, and 50 μl aliquots were dispensed in the 96-well black polystyrene microtiter plates. The wells were incubated overnight at 4°C. Following the removal of the coating buffer and three washing cycles with buffer B, a second coating was done using 100 μl of 20 g/l BSA solution. After washing, 20 μl sample or standards (over a range of 0.2–62.5 pg per well) were added in five duplicates to the assay wells. 30 μl anti-CAP monoclonal antibody (diluted to 1 μg/ml with buffer C) was added to each well. The wells were incubated with gentle shaking for 30 minutes at room temperature and then washed three times with 200 μl of PBST (buffer B). After washing three times again, 50 μl of horseradish peroxidase-IgG diluted in assay buffer (1:4000 from the stock solution) was dispensed and incubated for another 30 min at room temperature. After three washing cycles, chemiluminescent development was carried out by adding 50 μl of SuperSignal®

substrate solution. The intensity of the light emission was measured at 425 nm and the results were expressed in relative light units (RLU).

Chloramphenicol (CAP) concentration values were calculated by interpolation from the calibration curve, where the bound enzyme activity, expressed as the logit of the ratio (in percent) between CAP signal at each concentration of CAP (B) and the bound activity in the absence of unlabeled CAP (B0) was plotted against the log of CAP concentrations. The calibration curve is fit to a four-parameter equation, given by:

$$B/B_0 = (A - D)/[1 + (x/C) B] + D$$

where: A, D – the asymptotic B/B0 values as logAb → 0 and logAb → ∞, respectively

　　　B – related to the slope

　　　C – the predicted x value at the midpoint of the calibration curve (representing the sensitivity)

Optimisation of the parameter for ic-CLEIA

The optimisation for the competitive time. The primary antibody and the CAP series standard solutions were dispensed in the wells of microtiter plates at room temperature, and incubated for 10, 20, 30, 45 and 60 min, respectively.

To determine the optimum concentration of Tween-20. The antibodies (including the primary and the second antibody) were diluted with PBS containing different percents of Tween-20 (1, 0.5, 0.1, 0.05, 0.01 and 0.005%); the other steps were as previously described.

To determine the optimum ion strength of PBS solution. The antibodies (including the primary and the second antibody) were diluted with PBS of different concentration (1.0, 0.4, 0.2, 0.1, 0 mol/l) with 0.05% Tween-20; the other steps were as previously described.

To determine the optimum pH of PBS solution. The antibodies (including the primary and the second antibody) were diluted with PBS of different pH (4.0, 5.0, 6.0, ~10.5) with 0.05% Tween-20; the subsequent steps were as previously described.

Precision and accuracy. Sample preparation: in the case of whole shrimps, the heads, chitinous shells and body appendages were removed and the remainders were cut into pieces and treated with household stirring machine. Then they were preserved by airtight sealing at –20°C. Then the clean shrimps were put in the 10 ml homogenate

apparatuses labelled 1, 2, 3, 4 or 5 g/apparatus. Further, 8, 7.95, 7.9 and 7.6 ml ethyl acetate were added.

For the spiking studies, homogenised shrimps were used. Shrimp samples were spiked at 0 (negative control), 0.1 (low pool), 2 (medium pool) and 8 (high pool) ng/g level by adding a 100 ng/ml CAP standard stock solution before the beginning of the extraction procedure described.

The shrimp samples were homogenised for 30 min at room temperature, and then transferred into four centrifuge tubes of 15 ml each. The homogenisation apparatus was washed with 2 ml ethyl acetate and the washings were combined with the shrimp homogenates. The tubes were centrifuged at 4000 rpm for 10 min at room temperature. After the removal of the supernatant, the residues were dried under a stream of nitrogen. The residues were then redissolved in 2 ml *n*-hexane, 1 ml PBS with 0.02 (v/v) Tween-20 (PBST) was added, the mixtures were shaken vigorously and centrifuged as described previously. The supernatants were transferred to four clean tubes. The precipitates were redissolved with 1 ml *n*-hexane, centrifuged, and the supernatants were combined.

The spiked shrimp samples at low, medium and high concentrations corresponding to 0.1, 2, 8 ng/ml were added in six duplicates to the assay wells. The precision was evaluated by assaying the same group of spiked samples on four different days, followed by the calculation of the inter- and intra-coefficients of variation (CV). The accuracy was evaluated by measuring the percentage recovery.

RESULTS AND DISCUSSION

The optimum competitive time

The optimum competitive time was determined by assaying different competitive times after CAP
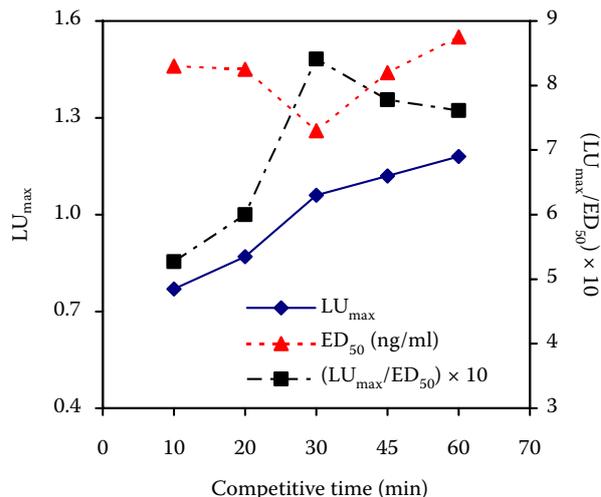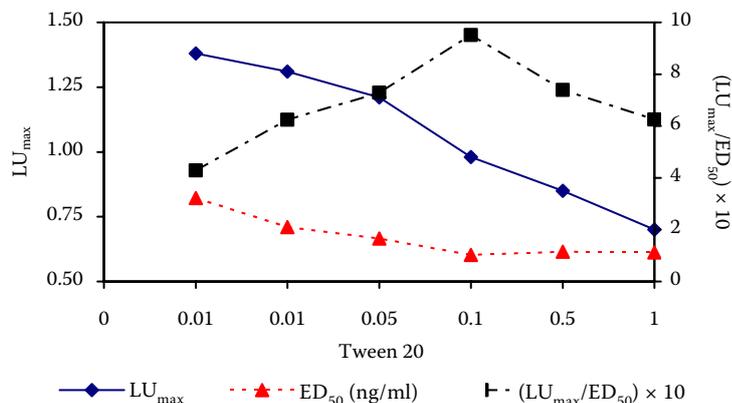

Figure 1. Variation curve of the ic-CLEIA parameters with competitive time

standard solution and primary antibody were dispensed in the wells of the microtiter plates at room temperature. The variation curves of the ic-CLEIA parameters with competitive time show that, between 10 and 30 min, the max light units (LU_{max}) increase with time while ED_{50} decreases; between 30 and 60 min, LU_{max} is stable, while ED_{50} increases with the time increase (Figure 1). Thus, 30 min is the optimum competitive time, ED_{50} being at minimum, while LU_{max}/ED_{50} at maximum.

The optimum concentration of Tween-20

As shown in Figure 2, when the concentration of Tween-20 is lower than 0.1%, LU_{max} and ED_{50} increase with the decreasing Tween-20 concentration. When the concentration of Tween-20 is higher than 0.1%, LU_{max} decreases, while ED_{50} does not change much; so, 0.1% is the optimum concentration of Tween-20 for the maximal signal and the least non-specific binding.

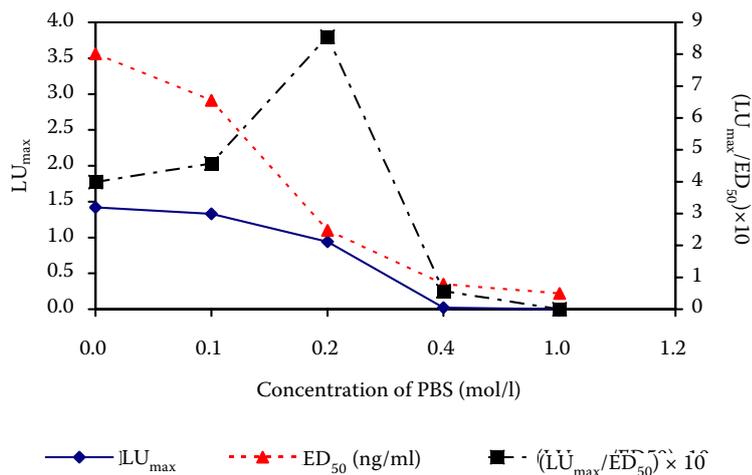Figure 2. The influence of the Tween-20's concentration on the ic-CLEIA for CAP

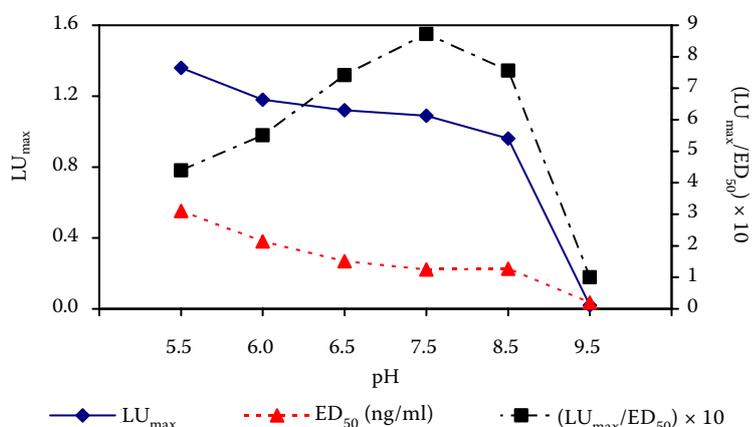Figure 3. The influence of the ionic strength of PBS on the ic-CLEIA for CAP

Figure 4. The influence of PBS' pH on the ic-CLEIA for CAP

The optimum ionic strength of PBS

The influence of the ionic strength of PBS on the ic-CLEIA is shown in Figure 3. It can be concluded from the figure that the concentration of PBS affects the assay performance. Both LU_{max} and ED_{50} decrease with the increasing concentration of PBS. At the concentration of PBS of 0.2 mol/l, LU_{max}/ED_{50} is maximum, indicating that 0.2 mol/l is the optimum concentration for PBS.

The optimum pH for PBS

As pH affects the ionic strength of CAP standard solution and of the other reagents, the acidity of the assay medium has a great effect on the detection limit and the non-covalent reaction between the antibody and the antigen. The curves in Figure 4 obtained at several pH values show that at pH values below 7.5, ED_{50} decreases quickly from 3.1 to 1.25 with the decreasing pH. Although the value of LU_{max} also decreases with decreasing pH, the decrease is small. At pH below 5.5, the reaction is entirely inhibited. The curves indicate that the

system tolerates better slightly acidic media than alkaline. The assay performance appears to be only moderately affected by changes in pH between 6.5 and 9, and to have an optimum around 7.5.
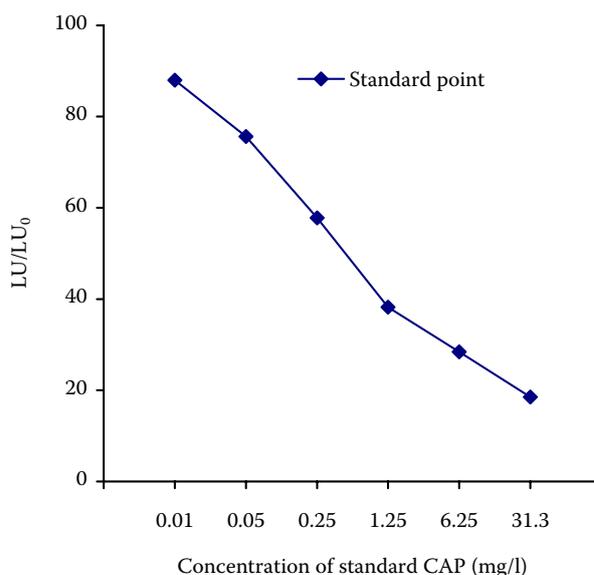

Figure 5. Optimised calibration curve for the ic-CLEIA of CAP

Table 1. Results of precision and accuracy assays

Samples (ng/ml)	Mean ± SD (ng/ml)	CV (%)	Recovery (%)
Intraassay ($n = 6$)			
0.1	0.123 ± 0.01	8.1	123.0
2	2.26 ± 0.11	4.9	113.4
8	8.05 ± 0.31	3.8	100.6
Interassay ($n = 4$)			
0.1	0.118 ± 0.014	11.9	118.0
2	2.17 ± 0.18	8.3	108.5
8	7.61 ± 0.38	5.0	95.1

ic-CLEIA calibration curve

Under the optimised conditions, the representative dose-response curve of the mean standard value is shown in Figure 5. The ED_{50} is 0.47 ng/ml, the detection range (calculated as the concentration responding to 20% and 80% LU/LU_0) is variable from 0.03 to 23.7 ng/ml, the detection limit (LOD), calculated as the 90% value of LU/LU_0, is 0.008 ng/ml. The parameter described is responding to the dot line respectively.

Precision and accuracy

By assaying the same group of spiked samples on four different days, the inter-assay was found to be 5.0% for 8 ng/ml, and 12% for 0.1 ng/ml. Being all below 15%, it indicted that the precision for ic-CLEIA can be accepted. The accuracy ranging from 100.6 to 123.0% for CAP concentrations from 0.1 to 8 ng/ml also indicted that the accuracy for ic-CLEIA is satisfactory for the actual assay (Table 1).

CONCLUSION

The method of CLEIA has the advantages of a better sensitivity, a lower antibody requirement, and a shorter assay time as compared to colored ELISA in the quantitative determination of the chloramphenicol residues in aquatic product.

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