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Development of an indirect competitive ELISA for detection of danofloxacin residue in milk

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Abstract

In order to develop the immunoassay method of detecting residue of Danofloxacin (DFLX) in milk, DFLX was used as a hapten which was coupled with Bovine serum albumin (BSA) and DFLX-BSA was formed. Then the rabbits were immunized with it and the specificity antibody against DFLX was obtained. The assay performance was improved by use of a coating antigen (DFLX-OVA), which was a conjugation of the DFLX and Ovalbumin (OVA). Finally, the determination of DFLX residue by Indirect Competitive ELISA (Ci-ELISA) was studied. The results of the experiment demonstrated that limit of detection (LOD) for DFLX was 29.24 µg/kg. Besides, the specificity antibody crossly reacted with the closely related FQNs, including Ciprofloxacin (CPLX), Ofloxacin (OFLX), Sarafloxacin (SALX) and Enrofloxacin (EFLX). The average recovery rate of standard substance (DFLX) is 91.45% within the range of 0.5~2 000 µg/kg in milk. The developed method was practicable to screen the residues of DFLX in milk.

Introduction

Danofloxacin (DFLX) is special fluoroquinolones (FQNs) antibacterial drugs in animals, it belong to the quinolone and fluoroquinolone family, which features broad-spectrum antibacterial activity based on the inhibition of bacteria DNA (Shojaee Aliabadi and Lees, 2003). Danofloxacin Mesylate is usually used in clinical and widely used in treatment of the respiratory disease, the genitourinary tract infections and mycoplasma disease of common animals. But the overdue use of the Danofloxacin resulted in residue in animal tissues, not only making pathogenic bacteria resistant against human, but also producing side effects. Therefore, governments and organizations enacted the Maximum Residue Limit (MRL) of Danofloxacin in the different parts of animal (Council Regulation (EEC), 1990; Commission Reguation (EC), 1999; Food and Agriculture Orgnization, 2003). The Danofloxacin MRLs in poultry, cows, sheep, goats and other animal parts and in milk are regulated in the Veterinary Drug Maximum Residue Limits in the Food of Animal Origin (No. 235) by China Ministry of Agriculture on Dec.24, 2002. Among them, the Danofloxacin MRL is 30µg/kg in milk (Ministry of Agriculture of the People's Republic of China,2002).

Most of analytical methods concerning FQNs were based on high-performance liquid chromatography (HPLC) (Toussaint B,2005). However, it was time-consuming, required expensive instruments and trained personnel. Therefore, establishing a rapid detection method of Danofloxacin residue in milk has to become to a new goal of scientific and technical workers.

The Immunoassay such as enzyme-linked immunosorbent assay (ELISA) is the most suitable methods for screening of drug residues in the veterinary field due to its rapidity, mobility and high sensitivity with deteetion limits in the nanogram(ng) range. In this paper, we successfully established the immunoassay methods for the DFLX, the immunoassay conditions of indirect competitive enzyme-linked immunosorbent assay (Ci-ELISA) was explored. The methods is inexpensive, time-saving and suitable for on-site testing.

Danofloxacin was taken as research object in this study. It was used as a hapten which were coupled with BSA and OVA respectively by N-hydroxyl amber imide possess active ester (NHS) method, and DFLX-BSA and DFLX-OVA were formed. Then the rabbits were immunized with DFLX-BSA and the specificity antibody against DFLX was obtained. The study of Danofloxacin residue immunology screening test methods for milk will lay a foundation on Danofloxacin residue of milk Assay Kit and rapid test strip.

Materials and Methods

Materials

Danofloxacin mesylate reference substance

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(DFLX, 99.7%), Sarafloxacin reference substance (SALX,99.6%), Norfloxacin reference substance (NFLX,99.8%), Ciprofloxacin hydrochloride reference substance (CPLX,100.0%), Lomefloxacin hydrochloride reference substance (LFLX,99.8%), Pefloxacin mesylate reference substance (PFLX,99.9%), Enrofloxacin reference substance (EFLX,100.1%), Ofloxacin reference substance (OFLX, 99.0%) were purchased from China Institute of Veterinary Drug Control. Bovine serum albumin (BSA, MW 67 000), Ovalbumin (OVA, MW 45 000), Ethyl[3-(dimethylamino) propyl]carbodiimide hydrochloride (EDC.HCL), O-phenylenediamine (OPD), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) were from Sigma Company, and Dimethylformamide (DMF) from Genview Company, N-hydroxysuccinimide (NHS), from Cxbio Biotechnology Company, Horseradish peroxidase labeled goat anti-rabbit IgG from Zhongshan Goldenbridge Biotechnology Co.,LTD. Goat serum was self-made in the laboratory; Phosphate-buffered saline pH 7.4 (PBS): 8.0 g/L of NaCl, 0.2 g/L of $NaH_2PO_4 \cdot 2H_2O$, 2.9 g/L of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g/L of KCl. PBS containing 0.05% tween 20 (PBST) for washing steps. The stopping solution was 2M H_2SO_4. Other reagents were all of analytical grades, made in China.

UV spectra and visible-region absorbance was measured by a 756 PC spectrophotometer (Shanghai Spectral Instrument Co.LTD). Absorbance value obtained in ELISA was read and recorded with a Bio-TEK ELX 800 ELISA Microplate Reader (in America), Ninety-six well-polystyrene microtitration plates were bought from NUNC Co. in Denmark. 2 -2.5 kg adult female rabbits were bought from the Experiment Center of Hebei North University.

Synthesis of immunogen and coating antigen

Synthesis of immunogen and coating antigen were made by N-hydroxyl amber imide possess active ester (NHS) method (Tijssen P, 1985), with the following minor modifications. A total of 40.0 mg DFLX, 20.0 mg NHS and 25.0 mg EDC.HCL dissolved completely in 2.0 mL DMF. The mixture solution was stirred for 24.0 h at room temperature in dark, and DFLX reaction liquid was got. 100.0 mg BSA dissolved fully in 6 mL PBS (0.01 mol/L, pH 7.4). Then DFLX reaction liquid was added to the BSA solution slowly being stirred, followed by stiring for 3.0 h at room temperature. Finally, the reaction mixture was dialyzed under stirring against PBS for 3 d with PBS solution (changing it three times a day) to remove the unconjugated hapten. The dialysates were centrifuged at 12 000 rpm for 30 min at room temperature, and the supernatant fluid was collected, which was DFLX-BSA immunogen. Then it was repacked and stored at -20°C. Replace BSA with OVA and do as above, and make a preparation of DFLX-OVA coating antigen.

Scanning identification of complete antigen by ultraviolet spectrum

BSA, OVA, DFLX, DFLX-BSA and DFLX-OVA with PBS were diluted respectively. In 200~400 nm UV light spectrum the materials were respectively scanned to confirm whether they had been coupled (Xinjian Chen and Meiying Chen, 1998). Meanwhile, the calculation of vector protein and the hapten coupling ratio was referred to that by Hongxia Yu (Hongxia and Shuming, 2005).

Animal immunization

With reference to the Dong's method (Dong *et al.*, 2009), three adult female New Zealand white rabbits were subcutaneously immunized by multi-point injection in the back with DFLX-BSA conjugate. FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every 14 days with 1.0 mg of immunogen and 5 times altogether, and blood samples were taken for ELISA identification from the marginal vein of the ear after 7 d of each immunization. Seven days after the final boost, all rabbits were exsanguinated by heart puncture. The blood was stored at 4°C overnight and then was centrifuged at 3 000 rpm for 15 min at room temperature, and the serum was collected. The crude serum was purified with saturated ammonium sulfate (SAS) precipitation method(Liping Zhu and Xueqing Chen,2000). Finally, the purified antibody was dispensed into suitable containers, and stored at -20°C.

Indirect ELISA method to determine the rabbit antibody titer of DFLX

With reference to the Tijssen's method(Tijssen P,1985) , the indirect ELISA method was applied 7 days after each immunization to determine the antibody titer and whether the antibody was generated for immunogen. And select the high and stable titer antibody as experiment antibodies.

Selection of DFLX-OVA coating concentration and the antibody working concentration by Square Matrix titration method (Deshi et al., 2001)

The antigen coating concentration and the optimum working concentration of antibody were determined in accordance with the value of Optical

Density (OD_{490nm}) and the specific situation.

The choice of coating conditions for detection antigen (DFLX-OVA)

The appropriate coating condition was chosen with the antigen coated for 1.0 h at 37°C, 2.0 h at 37°C and overnight at 4°C respectively, by indirect ELISA test.

The choice of blocking time

The appropriate blocking time was selected under the condition of blocking coated ELISA plate with 5% goat blood serum in 0.5 h at 37°C, 1.0 h at 37°C, 2.0 h at 37°C and overnight at 4°C respectively, by indirect ELISA test.

The choice of competition reaction time

In order to reduce steps in the experiment, we selected competitive mode inside the operation step plate. The competition reactions were conducted in 0.5 h, 1.0 h, 1.5 h and 2.0 h at 37°C, respectively. Then compare with the different inhibition rates under four kinds of reaction times to choose the competition reaction time of the experiment.

Development of ELISA standard curves

Danofloxacin mesylate reference substance (DFLX) by 400 000,40 000, 4 000, 400, 40, 4 μg/kg dilution was used to establish the determination gradient, then the content of Danofloxacin was determined by the proposed Ci-ELISA method. The value of OD_{490nm} converted into %B/B0 (inhibition rate to the bottom I) value is y-coordinate, no DFLX inhibition of the OD_{490nm} value being B_0 and the corresponding DFLX inhibition of the OD_{490nm} concentration being B. With the corresponding DFLX concentration logarithm (Lg [DFLX]) for x-coordinate, the standard curve was drawn. Because of linear regression relation between $\%B/B_0$ value and Lg [DFLX], we can draw the regression curve equation and the correlation coefficient by means of regression analysis.

Determination of accuracy

We take the differences between the holes and those between the plates as the accuracy of the method. Different concentration standard samples being experimented for 4 times, the coefficient of variation (CV%) between the holes indicated the difference between the holes. The standard curve production being repeated for 3 times, coefficient of variation (CV%) between the plates indicated the difference between the plates (Deshi *et al*., 2001). CV % = SD (Standard Deviation) mean/average between holes (plates) binding rate × 100%

Determination of specificity

Seven different FQNs (SALX, NFLX, CPLX, LFLX, PFLX, EFLX, OFLX) were assessed for cross-reactivity with antibody of DFLX.

Determination of fortified recovery

A total of 500 mL of pure and fresh milk sample was centrifuged at 4°C with a speed of 10 000 rpm for 30 min, and the floated fat was discarded. In order to reduce the background interference, the extracted milk sample was diluted with PBS (total 10–fold dilution) and the concentration gradients with DFLX standard milk samples were prepared. These solutions were stored at -20°C and ELISA test was done within one week. The standard milk samples were treated according to "Development of ELISA Standard Curves" method. The fortified recovery is the calculated results (compared with the standard curve) divided by the actual dosage.

fortified recovery (%) = determination value (μg/kg) / added value (μg/kg) ×100%

Results

Hapten conjugation

The N-hydroxyl amber imide possess active ester (NHS) method was used to prepare immunogen and coating antigen. UV spectrometry was to determine the efficiency of the conjugation reaction. UV absorbances for DFLX-BSA, DFLX, and BSA were presented in Figure 1. The absorbance of DFLX-BSA gave a maximum peak at 277 nm compared with the 275 nm maximum peak for DFLX, and the 278 nm maximum peak for BSA, which indicated DFLX was successfully conjugated with BSA. The coating antigen DFLX-OVA gave a UV pattern similar to that of DFLX-BSA in Figure 2. Calculated according to the formula by Hongxia Yu (Hongxia and Shuming, 2005), DFLX-BSA coupling ratio was 10:1 and DFLX-OVA coupling ratio 18:1. Therefore, the antigen coupling can be further proved completely successful.

Determination of DFLX antibody titer results

In Figure 3, it is known that with the increase of number of immunization, the OD_{490nm} value of antibody also increased constantly, which proved the immunogen effective and the immune program correct. From 28 d immunization, OD_{490nm} value and P/N ratio of antibody constantly increased, but the extent of change is very small, especially for 42 d and 56 d with OD_{490nm} value and P/N ratio of antibody almost remaining the same, the result showed that the antibody reached a high level. Polyclonal antibodies

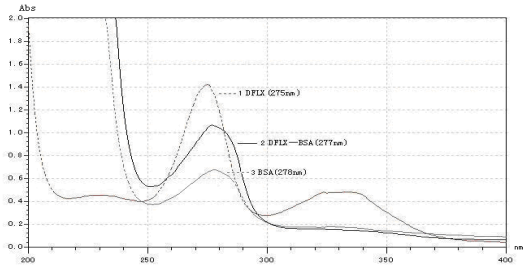

Figure 1. Utraviolet scan spectra for DFLX-BSA, DFLX, and BSA 1. DFLX solution 2. DFLX-BSA solution 3. BSA solution

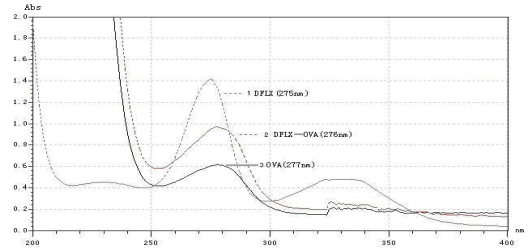

Figure 2. Utraviolet scan spectra for DFLX-OVA, DFLX, and OVA 1. DFLX solution 2. DFLX-OVA solution 3. OVA solution

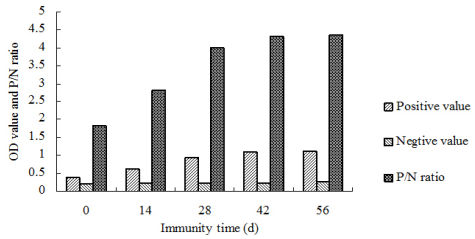

Figure 3. The relationship between immunity times and OD value of antibody

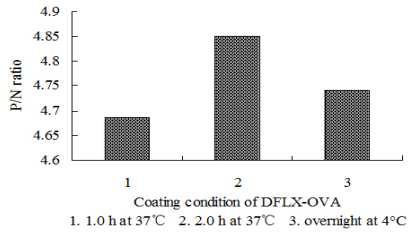

Figure 4. The P/N ratios in different coating condition of DFLX-OVA

with high specificity were obtained from the serum of rabbit immunized with purified recombinant protein. It demonstrated that antibody titer is stable and the blood from rabbits can be taken for following experiments.

The optimal choice of the DFLX-OVA coating concentration and the antibody working concentration

According to the coating antigen and the antibody consumption principle, the coating antigen and antibody concentration dilution degrees were chosen as the ideal work concentration respectively in the detection of around 1.0 (OD_{490nm} value) (Jie and Jian, 2005). So it is certain that optimal DFLX-OVA coating concentration was 0.25 μg/mL, the optimal

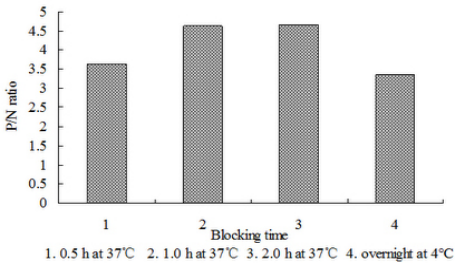

Figure 5. The effect of blocking time to ELISA

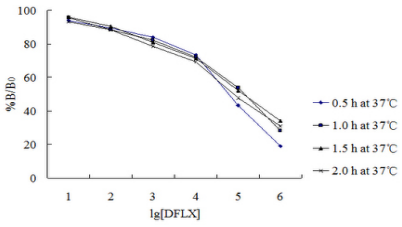

Figure 6. The selection of different competitive reaction time

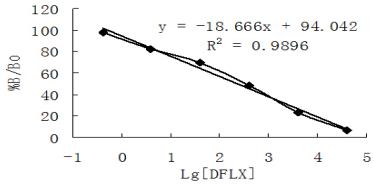

Figure 7. Standard curve for detection of DFLX by Ci-ELISA

concentration of antibody was 16 000 times diluted in the experiment system.

Coating conditions for detection antigen (DFLX-OVA)

As shown in Figure 4, when the DFLX-OVA coated the microtitration plates for 2.0 h at 37°C, P/N ratio was the highest, antibody positive value was the highest, and the coating effect was the best. So the optimum coating conditions of the experimental was 2.0 h at 37°C.

Blocking time

As shown in Figure 5, when microtitration plates were blocked 1.0 h and 2.0 h at 37°C, P/N ratios were almost the same. In order to save time, the reaction system blocked time was selected as 1.0 h at 37°C.

Competition reaction time

The results of the competition reaction time inhibition rate was shown in Figure 6. It is showed that the competition reaction time inhibition rate changed slightly, but overall, the competition reaction curve was more smooth and stable for 1.0 h at 37°C. So the competitive reaction time was chosen for 1.0 h at 37°C.

The establishment of the standard curve

The limit of detection for ELISA is I≥1.5-2 (Zhihong *et al.*, 1998), and the limit in the experiment

Table 1. The variability of within-assay and between-assay of the standard curve of Ci-ELISA

	Concentration of DFLX (μg/kg)						Average value (%)
	0.5	5	50	500	1 000	2 000	
Differences between the holes CV%	9.85	7.87	6.63	4.76	2.39	1.57	5.51
Differences between the plates CV%	15.31	12.18	9.63	7.65	4.82	2.39	8.66

Table 2. The intercross reaction of antibody of DFLX with other FQNs

Drug of competition	Cross-reactivity rate (%)
DFLX	100
CPLX	63.53
OFLX	60.67
SALX	35.97
EFLX	21.82
NFLX	14.79
LFLX	11.14
PFLX	<0.01

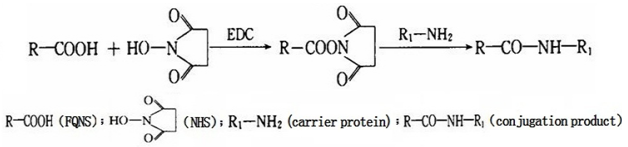

Figure 8. FQNs coupling principle diagram

is I=1.5, the inhibition rate $I=B_0/B$; and transform OD_{490nm} value into $\%B/B_0$, then make a regression analysis for Lg[FQNs] again. The results of Ci-ELISA was seen in Figure 7. The linear regression equation was $y = 18.666\ x + 94.042$ ($R^2 = 0.9\ 896$), which accorded with the judgment standard of the linear relationship, y being $\%B/B_0$ (inhibition rate I bottom), x being DFLX concentration logarithm Lg [DFLX]. When I equals 1.5, Log [DFLX] corresponds to 1.466 by the Figure 7, and the DFLX concentration is 29.24 μg/kg, namely, the limit of detection (LOD).

Accuracy

The variation coefficient of inside plates and between plate were shown in Table 1. The CV of the holes and plates ranged from 1.57% to 9.85% and 2.39% to 15.31%, respectively, the average were being 5.51% and 8.66% respectively. From the Table 1, it is clear that the hole and plate variation coefficient increased with the concentration reduce, and the CV of the plate had a little bigger than inside plate. It indicated that the method in the accuracy and repeatability is better at higher concentrations much more.

Specificity of the antibody

According to the ELISA test results, the cross-reactivity rate of the antibody against the standard solution of DFLX was set 100%, and the cross-reactivity rates of the antibody with other FQNs is shown in Table 2. It proves that the immunoassay is highly broad spectrum for DFLX.

Fortified recovery

From the Table 3, it is known that the recovery is higher from 5 to 2000 μg/kg. In this recovery concentration range, the added sample can be effectively recycled.

Table 3. The recovery rate of standard substance

Recovery rate (%)	Standard product concentration (μg/kg)						Average recovery rate (%)
	0.5	5	50	500	1 000	2 000	
DFLX	72.32	82.16	96.33	96.98	101.25	99.65	91.45

Discussion

Hapten conjugation

With a molecular mass of 357.38, DFLX is not able to stimulate the immune response in an animal for anti-DFLX antibody production and is non-immunogenic. To make it immunogenic, it must be conjugated to a carrier protein before its immunization. BSA and OVA are two of the mostly applied carrier proteins which are usually used for synthesizing immunogen and coating antigen respectively.

The synthetic pathways of complete antigens for immunization are presented in Fig. 8. As can be seen, FQNs contains a carboxylic acid. Thus, the immunogen and coating antigen can be prepared by the conjugation of the carboxylic acid group and an amino group of a carrier protein. In this study, the former linkage method was chosen in order to expose the functional part representing the feature of FQNs outward to increase the specificity of the antibody.

The standard curve fitting

A normal standard curve fitting, which has a certain function relation between the concentration and the absorbency value, and it can also build multiple regression relations. But because of the different analytes, the optimal curve fitting models of standard curve have certain differences. According to the References (Liguo *et al.*, 1998), the linear regression equation can be used to meet the needs of the experiment for ELISA detection and reduce the computational difficulty. So, in this experiment, the fitting form of linear regression is selected.

The differences between this method and the confirmatory methods

In order to reduce the detection cost for large quantities of samples, improve the detection efficiency, the screening analytical method is usually combined with the confirmatory analytical method by the detection departments. In detail, the screening analytical method was firstly applied to quickly analyze large actual samples, and then the positive samples were identified by the high performance liquid chromatographic (HPLC), mass spectrometry, thin layer chromatography, gas chromatography.

Conclusion

The limit of detection for the danofloxacin is 29.24 μg/kg in this method, which is below the Maximum Residue Limit (MRL) of Danofloxacin in milk set in China. Meanwhile, the antibody of DFLX can crossly react with other FQNs. Therefore, the method can also be used to screen other several FQNs residues. So, this method will be used to screen large-scale actual samples.

Acknowledgments

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