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Determination of Florfenicol Amine Residues in Animal Edible Tissues by an Indirect Competitive ELISA

JIN-E WU, CHAO CHANG,* WEN-PING DING, AND DONG-PING HE

College of Food Science and Engineering, Wuhan Polytechnic University, Wuhan 430023, People's Republic of China

Florfenicol (FF) is a broad-spectrum antibiotic used increasingly in aquaculture, livestock, and poultry to treat diseases. To avoid using labor-intensive instrumental methods to detect residues of FF in food and food products, a simple and convenient indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) method for florfenicol's major metabolite, florfenicol amine (FFA), was developed using a polyclonal antibody prepared in this study. FFA was covalently attached to carrier protein as immunogen by using the glutaraldehyde method. The antibodies obtained were characterized by an ELISA method and showed excellent specificity and sensitivity with the 50% inhibition values (IC_{50}) of 3.34 μg/L for FFA in PBS buffer. In the ELISA, sample extractions were performed by ethyl acetate/ammonium hydroxide (90 + 10, v/v) following combined acid hydrolysis of FF and its known metabolites. The limits of detection (LOD) calculated from the analysis of 20 known negative swine muscle, chicken muscle, and fish samples were 3.08, 3.3, and 3.86 μg/kg (mean + 3 SD), respectively. Recoveries of FFA fortified at the levels of 5, 50, 100, and 300 μg/kg ranged from 64.6 to 124.7%, with coefficients of variation of 11.3−25.8% over the range of FFA concentrations studied. Validation of the ELISA method with FFA-fortified swine muscle at the levels of 10, 50, 100, and 200 μg/kg was carried out using GC, resulting in a similar correlation in swine muscle ($r = 0.97$). The results suggest that this ELISA is a specific, accurate, and sensitive method, which is suitable for use as a screening method to detect residues of FFA in animal edible tissues.

KEYWORDS: Florfenicol amine; antibody; ELISA; residue; edible tissues

INTRODUCTION

Florfenicol (FF; **Figure 1**), a fluorinated analogue of thiamphenicol (TAP), is a synthetically produced broad-spectrum antibacterial agent specifically developed for veterinary use. Due to the ban of the use of chloramphenicol (CAP) in food-producing animals, florfenicol is used increasingly in aquaculture, livestock, and poultry to treat diseases (*1*−*6*). Although FF is a more secure drug than chloramphenicol, its use in animal husbandry has the potential to result in the presence of residues in tissues and the increased emergence of resistance of pathogenic bacteria that could have potential health risks to humans (*7*). Nowadays, antibiotic resistance has become a global threat because existing antibiotics are becoming increasingly ineffective in combating microbial infections in humans. To ensure the existence of FF antibiotics, the maximum residue limits (MRLs) in various tissues are fixed by many countries or organizations (*8*−*10*) (**Table 1**).

Reports have shown that FF is partly transformed into florfenicol amine (FFA; **Figure 1**), florfenicol oxamic acid (**Figure 1**), and florfenicol alcohol (**Figure 1**) in animal bodies after administration. Although their ratios are different in different species, FFA is found at the highest level of all the metabolites in mostly food animals (*10*−*13*). The present MRLs of FF in edible tissues are targeted to detecting the sum of FF and FFA. Florfenicol metabolism studies demonstrate that nonextractable residues of FF are predominant in edible tissues in poultry, swine, and cattle, although they are much less significant in salmon (*10*−*14*). Acid hydrolysis of these nonextractable residues yields a significant amount of FFA. Florfenicol and known metabolites of florfenicol are also converted to FFA by acid hydrolysis (*15*). Therefore, FFA is defined as one of the FF residue markers by many countries or organizations.

Many different methods have been described for the determination of FF in animal tissues, including liquid chromatography (*15*−*17*), LC-MS (*18*), gas chromatography (*19, 20*), and GC-MS (*21*). Wrzesinski et al. described an LC method for the determination of FFA in fish tissues by using acid hydrolysis (*15*). Van de Riet et al. reported an LC-MS method for the simultaneous determination of FF, FFA, CAP, and TAP in farmed aquatic species (*18*). Zhang et al. developed a GC method with a microcell electron capture detector for the simultaneous determination of FF and FFA in fish, shrimp, and

* Author to whom correspondence should be addressed (telephone 0086-27-83924790; fax 0086-27-83924790; e-mail changchao2000@yahoo.com.cn).

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swine muscle (*20*). Tomoko et al. described a GC-MS method for the simultaneous determination of FF, CAP, and TAP in fish tissues (*21*). Although these methods can produce satisfactory results for detecting FF, these instrumental analytical procedures usually are not fit for high-capacity screening analysis of FF. There is now an urgent need for a rapid, high-capacity, and sensitive screening method for FF residues to avoid using labor-intensive instrumental methods. Indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has become the most popular method for the detection of drugs in animal tissues due to its high sensitivity, simplicity, and ability to screen a large number of small-volume samples.

Although commercially the ELISA kit to detect residues of FF in animal tissues is available from Shenzhen Lvshiyuan Biotechnology Co., Ltd. (Shenzhen, China), its target analyte is florfenicol itself rather than residue marker FFA. From previous studies, it was reported that quantification of FFA in extracts of hydrolyzed tissues resulted in a more accurate determination of total florfenicol-related residue levels than only quantification of FF in simple solvent extraction (*15*). However, there are no reports of research to develop immunoassays to detect residues of FFA. Therefore, it is of great importance to have immunoassays to detect FFA residues. This leads us to the current study aiming to develop a reliable ELISA method for determination of FFA residues in animal tissues.

This paper describes an ELISA method for the detection of FFA residues following extracts of hydrolyzed tissues. In this study, the polyclonal antibodies produced with the immunogens FFA−BSA are highly specific and sensitive to FFA. This ELISA method has a tendency to incorporate into the ELISA test kit, which would provide a reliable ELISA method for screening analysis of FFA residues.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), ovalbumin (OVA), goat anti-rabbit IgG−horseradish peroxidase, 3,3′,5,5′-tetramethylbenzidine (TMB), glutaraldehyde, complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were supplied by Sigma-Aldrich (St. Louis, MO). Florfenicol (FF), thiamphenicol (TAP), and chloramphenicol (CAP) were purchased from Hubei Hongjing Chemical Co., Ltd. (Yingcheng, China). Florfenicol amine (FFA) was purchased from Shanghai Caienfu Technology Co., Ltd. (Shanghai, China). Polyoxyethylenesorbitan monolaurate (Tween-20) and *N*,*N*-dimethylformamide (DMF) were purchased from Linfei Biotec Co., Ltd. (Wuhan, China). All other chemicals were of analytical grade. New Zealand white rabbits were supplied by Hubei Centers for Disease Prevention and Control (Wuhan, China). ELISA was measured by enzyme immunoassay microplate reader MagellanCE 2.5 (Sunrise, Austria). GC analyses were performed by using an Agilent GC 6890 series (Agilent, Palo Alto, CA). UV−vis spectra of conjugates were obtained by Aglient UV−vis spectrophotometer 8453.

Preparation of FFA Conjugates. FFA was covalently attached to BSA to be used as immunogen or OVA to be used as coating antigen according to a glutaraldehyde method (*22, 23*). Glutaraldehyde was a cross-linker for preparation of FFA conjugates. In this procedure, 24.7 mg (0.1 mmol) of FFA was added to 136 mg (0.002 mmol) of BSA dissolved in the solution of 15 mL of PBS (0.1 M, pH 7.4) and 8 mL of DMF. Subsequently, 0.15 mL of glutaraldehyde (25%) was added dropwise to the solution. The conjugation mixture was then stirred at 4 °C for 4 h and centrifuged for 10 min (5000 rpm). The supernatant mixture was purified by exhaustive dialysis against PBS (0.1 M, pH 7.4) for 6 days and dried in vacuum at −70 °C to give immunogen powders. These powders was stored at −20 °C and diluted to appropriate concentration before use. The coating antigen conjugate was prepared by conjugation FFA with OVA as described above. Finally, UV−vis spectral data (**Figure 2**) supported the structures of the final conjugates. The hapten density (the number of hapten molecules per molecule of protein) of conjugates was estimated directly by mole absorbance ε: hapten density $= (\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}})/\varepsilon_{\text{hapten}}$. Molar ratios of approximately 23 and 17 were obtained for FFA−BSA and FFA−OVA conjugates, respectively.

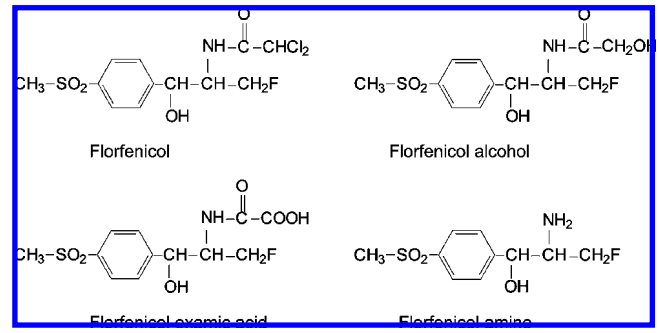

Figure 1. Chemical structures of florfenicol and its major metabolites florfenicol alcohol, florfenicol oxamic acid, and florfenicol amine.

Table 1. MRLs for Sum of FF and FFA in Swine Rissues (Micrograms per Kilogram)[a]

tissue	PRC[b]	EU[b]	USA[b]
muscle	300	300	200
liver	2000	2000	2500
kind	500	500	

[a] MRLs for other tissues were not shown, which were >100 μg/kg. [b] PRC, People's Republic of China; EU, European Union; USA, United States of America.

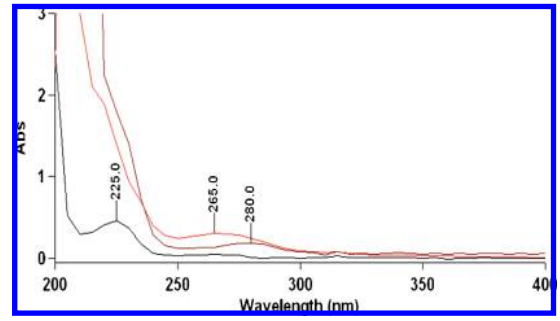

Figure 2. Ultraviolet absorbance spectra of BSA, FFA, and FFA−BSA. BSA, FFA, and FFA−BSA were dissolved in water. Following the result, the wavelengths of maximum absorbance of BSA, FFA, and FFA−BSA were 280, 225, and 265 nm, respectively. This result suggested that hapten FFA would be likely to conjugate to the protein carrier successfully.

Production of Polyclonal Antibodies. Polyclonal antibodies were raised by multiple-site injection of FFA−BSA conjugates into New Zealand white rabbits, using an immunization approach similar to that described in Chang et al. (*24*). Briefly, four schemes (three rabbits per scheme) used for immunizing are shown in **Table 1**. Scheme A employed 1 mg of immunogen per injection with booster injections every 2 weeks. Scheme B employed 1 mg of immunogen with booster injections every 4 weeks. Scheme C employed 0.5 mg of immunogen with booster injections every 2 weeks. Scheme D employed 0.5 mg of immunogen with booster injections every 4 weeks. At 10 days after each immunization, the antisera were tested by checkerboard (*25, 26*). When the titer of antisera was stable, the last injection was then given. One week later, whole blood of the each rabbit was collected individually. After being separated from blood cells by centrifugation (10000 rpm, 30 min), the antisera were purified by ammonium sulfate precipitation and then dried in vacuum at −70 °C to give antibody powders. These powders was stored at −20 °C and diluted to appropriate concentration before use. The stock solution of antibody (5 mg/mL) was stored at−20 °C in the presence of 50% glycerol and 0.1% NaN_3.

Indirect Competitive ELISA Procedure. The protocol for ic-ELISA was similar to that described previously (*24, 26*). In general, 96-well immunoplates were coated with 100 μL of FFA−OVA

conjugate (100 μg/L) in carbonate buffer (0.1 M, pH 9.6) overnight at 4 °C. The plates were washed three times with PBST and treated with 200 μL of 2% OVA in PBS for 1 h at room temperature. After 1 h of incubation, the plates were washed three times with PBST, and 50 μL of the antibody against FFA (250 μg/L) and 50 μL of various concentrations of standard FFA or the samples were added to each well for 1 h at 37 °C. After 1 h of incubation, the plates were washed three times with PBST and then reacted with 100 μL of HRP-labeled antirabbit IgG (1:10000 dilution in PBS) for 1 h at 37 °C. The plates were washed four times with PBST, and then 100 μL of TMB substrate solution was added to each well and incubated for 30 min at room temperature in the dark, followed by the addition of stopping solution (2 M, H_2SO_4). The absorbance at 450 nm was measured using a microplate reader. Sigmoid curves were fitted to a logistic equation from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined. A linear dose−response standard curve was prepared by plotting log[FFA] versus percent binding (B/B_0), where B and B_0 were the absorbance of the analyte at the standard point and at zero concentration of the analyte, respectively.

Assessment of Antibody Characterization. Assessment of antibody characterization was carried out using antibody titer, sensitivity, and cross-reactivity (CR). The titer of antibody was tested by checkerboard titration. In the checkerboard titration experiment, the titer at the well with an absorbance of 1.0 was defined as the best antibody titer. IC_{50} and CR (%) values were used to assess antibody sensitivity and cross-reactivity, respectively, which were determined by the ic-ELISA procedure described above. The cross-reactivity (CR) values were calculated as follows: (IC_{50} of FFA/IC_{50} of competitor) × 100.

Optimization of ic-ELISA. Assay optimization was performed using FFA as the competitor analyte. Various working concentrations of antibody Ab-rabbit$_{10}$ (125, 250, and 375 μg/L) and coating antigen (50, 100, and 200 μg/L) were studied sequentially to improve the sensitivity of the immunoassay using the ic-ELISA procedure as described above. The main criteria used to evaluate immunoassay performance were IC_{50}, slope, and R^2 of their linear equation.

Sample Preparation Procedure. The sample preparation procedure was performed as a modification of that described by Wrzesinski et al. (*15*). Briefly, 2 g of the sample homogenate (swine muscle, chicken muscle, and fish) was weighed into glass centrifuge tubes. Fortification samples were prepared at this point by adding various concentrations of FFA standard solution to the control tissues. Eight milliliters of hydrochloric (6 M) was added to each tube. Samples were shaken for 5 min and then placed in a shaking water bath at 100 °C for 3 h. After hydrolysis, the hydrolysates were extracted with 10 mL of ethyl acetate, and the mixture was centrifuged for 10 min at 5000 rpm. The ethyl acetate (upper) layer was removed by aspiration and discarded, and the supernatant hydrolysates were transferred to a clean tube. The samples were adjusted to pH 7.5 with 6 M NaOH, followed by extraction with 15 mL of ethyl acetate/ammonium hydroxide (90 + 10, v/v) three times. The extracts were combined in the flask and evaporated to dryness on a heating block at 40 °C under nitrogen. They were redissolved in 20 mL of PBS and used for ELISA detection.

Validation. The validation of the ELISA was the similar to that described in a previous paper (*24, 27*). Briefly, immunoassay validation was carried out using limit of detection (LOD), recovery (percent) of the fortified FFA, and coefficients of variation (CV). The standard FFA solution was diluted in PBS to obtain a five-point standard curve (0.5, 1.5, 4.5, 13.5, and 40.5 μg/L). The determination of LOD was based on 20 blank samples accepting no false-positive rates (average + 3 SD) (*24*). Twenty different swine, chicken, and fish samples were purchased in retail outlets in Wuhan, which had previously been proved to be free of FFA using GC determinations in Hubei Province Key Laboratory of Primary Products Processing and Transformation (Wuhan Polytechnic University, China). The recovery was determined by the analysis of the above samples fortified with FFA at the levels of 5, 50, 100, and 300 μg/kg and calculated as follows: (concentration measured/concentration fortified) × 100. The precision of the ELISA was analyzed by repeated determination of the samples' intra-assay and interassay coefficients of variation at the levels of 5, 50, 100, and 300 μg/kg for 5 days.

Table 2. Comparison of Immunization Schemes, Titers, and IC_{50} Results of Polyclonal Antisera Produced with FFA−BSA Immunogen

antibody	scheme	immunogen dose (mg)	interval (weeks)	titer	IC_{50} (μg/L) for FFA
Ab-rabbit$_1$	A	1	2	16000	24.19 ± 2.57[a]
Ab-rabbit$_2$	A	1	2	no response	no response
Ab-rabbit$_3$	A	1	2	8000	>100
Ab-rabbit$_4$	B	1	4	16000	10.74 ± 0.91
Ab-rabbit$_5$	B	1	4	16000	8.13 ± 0.76[a]
Ab-rabbit$_6$	B	1	4	32000	12.37 ± 1.15
Ab-rabbit$_7$	C	0.5	2	16000	11.56 ± 0.93
Ab-rabbit$_8$	C	0.5	2	32000	9.84 ± 0.74[a]
Ab-rabbit$_9$	C	0.5	2	16000	17.64 ± 1.58
Ab-rabbit$_{10}$	D	0.5	4	64000	3.34 ± 0.21[a,b]
Ab-rabbit$_{11}$	D	0.5	4	32000	8.73 ± 0.69
Ab-rabbit$_{12}$	D	0.5	4	32000	6.84 ± 0.54

[a] Sigmoid curves for the most sensitive antibody in each scheme are illustrated in **Figure 3A**. [b] FFA standard curve for Ab-rabbit$_{10}$ is shown in **Figure 3B**. Our efforts were focused on ELISA based on the most sensitive antibody Ab-rabbit$_{10}$.

Comparison of ELISA and Reference Method. The performance of the new ELISA method was compared with that of the GC method using FFA-spiked swine muscles at the levels of 10, 50, 100, and 200 μg/kg with three replicates per concentration. The same samples were subjected to this ELISA procedure and GC analysis according to the procedure of Zhang et al. (*20*), respectively. The GC system consisted of a microcell electron capture detector (μECD) and a splitless injector (Agilent). Separation was accomplished on a methyl siloxane capillary column (250 × 0.25 μm). The flow rate was 40.0 mL/min, inlet temperature was 270 °C, and injection volume was 5 μL. The ELISA procedure was carried out as described above.

Analysis of Field Samples. For this experiment, 20 samples (fish and swine muscle) were collected from retail outlets in Wuhan. The samples were homogenized and stored at −20 °C until use. Each sample was divided into two portions. One was analyzed by the ELISA and the other by GC. GC analysis of FFA was done according to the method of Zhang et al. (*20*).

RESULTS

Characterization of Polyclonal Antibodies. To assess characterization of the antibodies produced with FFA−BSA conjugate, the antibody titer, sensitivity, and cross-reactivity (CR) were determined by checkrtboatd titration or the ic-ELISA procedure described above. Comparison of the titer and IC_{50} values for the anti-FFA antibodies in various immunization schemes was shown in **Table 2**. Results indicated that the antibodies displayed a high level of affinity and sensitivity for FFA, with the favorable titers and IC_{50} values, except for Ab-rabbit$_2$ and Ab-rabbit$_3$. Sigmoid curves for the most sensitive antibody in four immunization schemes are illustrated in **Figure 3A**. It was found that Ab-rabbit$_{10}$ in immunization scheme D was the most sensitive antibody, which our efforts were focused on. It could be seen from sigmoid curves that the LOD for Ab-rabbit$_{10}$ was 0.1 μg/L. The FFA standard curve based on PBS solution for Ab-rabbit$_{10}$ is shown in **Figure 3B**. We observed that in the range of 0.5−40.5 μg/L, the graph between "y" and "x" was linear (**Figure 3B**), and the regression equation was obtained ($y = -39.301x + 70.512$, $R^2 = 0.9855$). The specificity of antibody Ab-rabbit$_{10}$ was evaluated with florfenicol, thiamphenicol, chloramphenicol, and various other veterinary drugs, which was determined by measuring their IC_{50} values. Chemical compounds and their corresponding cross-reactivities are shown in **Table 3**. Antibody Ab-rabbit$_{10}$ showed 10.88% CR with florfenicol, 4.33% CR with thiamphenicol, and 1.56% CR with chloramphenicol, which exhibited negligible CR with various other veterinary drugs (<0.01%). The results presented in **Tables 2** and **3** and **Figure 3** demonstrate that antibody Ab-rabbit$_{10}$ was highly specific and sufficiently sensitive to FFA.

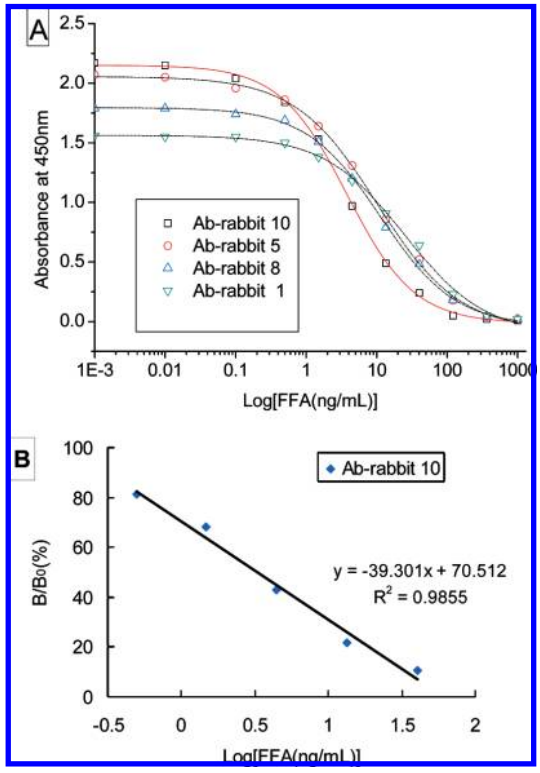

Figure 3. Competitive indirect ELISA curve for FFA: (**A**) sigmoid curves for the most sensitive antibody in four immunization schemes (log[FFA] was logarithm concentration of FFA); (**B**) FFA standard curves for Ab-rabbit$_{10}$ transformed from **A** in the range of 0.5−40.5 μg/L. B/B_0 was the normalized response relative to the zero standard. The regression curve equation of the antibody Ab-rabbit$_{10}$ was $y = -39.301x + 70.512$ ($r = 0.99$).

Table 3. Cross-Reactivities of the Most Sensitive Antibody Ab-Rabbit$_{10}$ with Various Drugs

competitor	IC_{50} (μg/L)	cross-reactivity[a] (%)
FFA	3.53	100
florfenicol	32.44	10.88
thiamphenicol	81.46	4.33
chloramphenicol	225.63	1.56
furazolidone, tetracycline, ampicillin, enrofloxacin, sulfamethazine, metronidazole	>100000	<0.01

[a] Cross-reactivity (%) = (IC_{50} of FFA/IC_{50} of competitor) × 100.

Optimum ELISA Conditions. In this section, various working concentrations of antibody Ab-rabbit$_{10}$ (125, 250, and 375 μg/L) and coating antigen (50, 100, and 200 μg/L) are studied for the selection of the optimum ELISA conditions using the ic-ELISA procedure as described above (*24*). The influence of various concentrations of coating antigen and antibody Ab-rabbit$_{10}$ on competition reaction is shown in **Figure 4**. **Table 4** summarizes the characteristic parameters (IC_{50}, slope, and R^2 values) of the ELISA method. Results demonstrated that the coating antigen concentration of 100 μg/L and the antibody Ab-rabbit$_{10}$ concentration of 250 μg/L were needed to get to the lower IC_{50} values and satisfactory slope and R^2 values. These conditions were fixed for the rest of the experiment.

Validation. The results of determination of 20 different blank samples are shown in **Table 5**. On the basis of the determination of 20 different blank samples, the LODs in swine muscle, chicken muscle, and fish were 3.08, 3.3, and 3.86, respectively. The results of recoveries and interassay variability coefficients

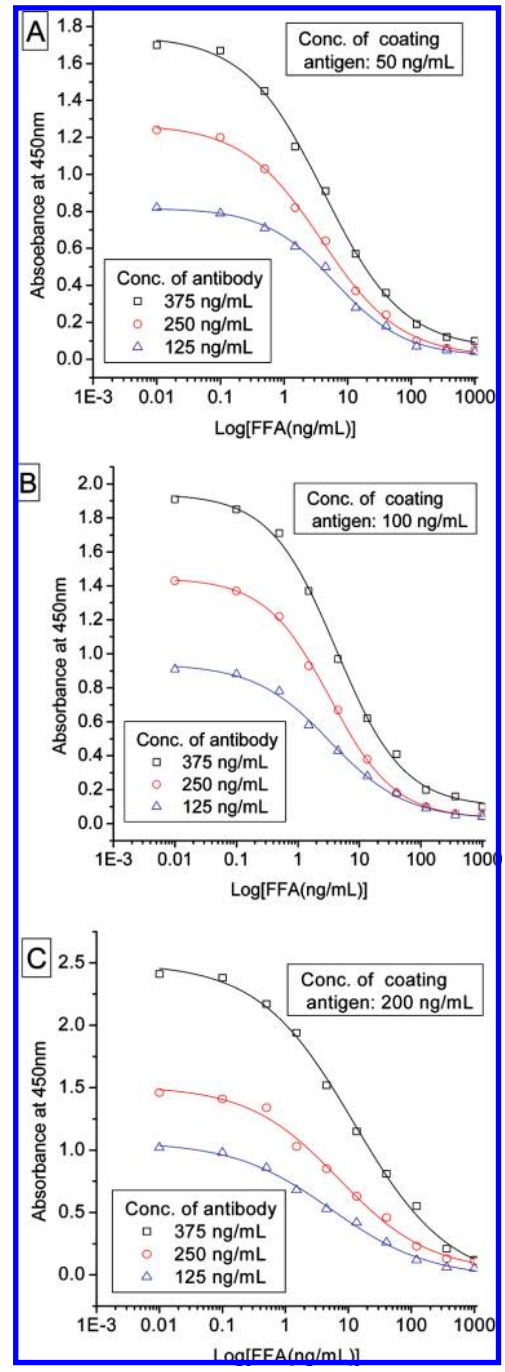

Figure 4. Influence of various concentrations of coating antigen FFA−OVA and antibody Ab-rabbit$_{10}$ on competition reaction. The concentrations of coating antigen FFA−OVA in graphs **A**, **B**, and **C** were 50, 100, and 200 ng/mL, respectively. The concentrations of antibody Ab-rabbit$_{10}$ were follows: 125 (△), 250 (○), and 375 (□) ng/mL.

of the above samples fortified with FFA at the levels of 5, 50, 100, and 300 μg/kg are presented in **Table 6**. The test was repeated five times with three replicates per concentration. When FFA was fortified at four levels in the above samples, the recoveries of FFA ranged from 64.6 to 124.7% with CVs of 11.3−25.8%.

Comparison of the ELISA and GC Analyses. Figure 5 demonstrates the performance of the ELISA in comparison with the confirmatory GC method for the determination of FFA in swine muscle. The samples were obtained from FFA-spiked swine muscles at the levels of 10, 50, 100, and 200 μg/kg with three replicates per concentration. **Figure 5** illustrates that the

Table 4. Influence of Various Concentrations of Coating Antigen FFA−OVA and Antibody Ab-Rabbit$_{10}$ on Parameters of ELISA Methods[a]

concn of coating antigen (ng/mL)	concn of antibody (ng/mL)	IC_{50} (μg/L) for FFA	slope	R^2
50	125	5.78 ± 0.49	−34.53 ± 0.43	0.98
	250	4.31 ± 0.42	−34.31 ± 0.38	0.99
	375	4.74 ± 0.39	−33.26 ± 0.63	0.99
100[b]	125	3.91 ± 0.37	−33.46 ± 0.52	0.98
	250[b]	3.49 ± 0.28	−36.82 ± 0.42	0.99
	375	4.91 ± 0.51	−35.56 ± 0.43	0.98
200	125	4.64 ± 0.45	−28.03 ± 0.37	0.99
	250	7.64 ± 0.68	−30.11 ± 0.51	0.98
	375	10.68 ± 0.96	−29.72 ± 0.38	0.99

[a] Each value is the mean ± SD ($n = 5$). [b] Results demonstrate that a coating antigen concentration of 100 ng/mL and an antibody Ab-rabbit$_{10}$ concentration of 250 ng/mL were needed to get to the lower IC_{50} values and satisfactory slope and R^2 values.

correlation coefficient for both methods was 0.97 (r) in the determination of swine muscle, with a regression coefficient of 1.18. The results imply a good agreement between FFA concentrations determined by the two methods and prove the reliability of the ELISA for the determination of spiked samples.

Analysis of Field Samples. Twenty samples collected from retail outlets in Wuhan were analyzed. The results are presented in **Table 7**. FFA was not detected in these samples, except for two swine muscles and three fish samples. It is shown in **Table 7** that the concentrations of FFA measured by the ELISA were lower than those by the GC method (148.4 and 56.2 μg/kg by ELISA, cf. 181.3 and 83.6 μg/kg by GC in swine muscle; and 922.3, 352.5, and 104.7 μg/kg by ELISA, cf. 1203.4, 443.8, and 151.3 μg/kg by GC in fish sample, respectively), but the negative results detected by the ELISA were coincident with those by the GC method. The results demonstrate this ELISA can be used as a screening method for the determination of FFA in real tissues without false negatives.

DISCUSSION

The design of specific haptens is the key step in a procedure of research on rapid immunoassay. To date, we do not find any reports of immunoassays for the determination of florfenicol or florfenicol amine in animal edible tissues, but the commercial ELISA kit with a rather lower LOD (1−3 μg/kg in various tissues) to detect residues of FF in animal tissues is available from Shenzhen Lvshiyuan Biotechnology Co., Ltd. Although its results of determination for FF are favorable, its target analyte is florfenicol itself rather than residue marker FFA. Florfenicol metabolism studies demonstrated that nonextractable residues of FF are predominant in edible tissues in poultry, swine, and cattle, although they are much less significant in salmon (*10*−*14*). Acid hydrolysis of these nonextractable residues in other species yields a significant amount of FFA, an extractable product and metabolite of FF (*10*−*13*). An acid hydrolysis step for the sample preparation has been developed by Wrzesinski et al. (*15*), which can convert FF and its related metabolites to FFA. These previous studies with florfenicol in cattle, poultry, swine, and salmon identify florfenicol amine as the marker residue. Quantification of FFA in extracts of hydrolyzed tissues results in a more accurate determination of FF in simple solvent extraction. Therefore, FFA is chosen as a hapten to generate specific antibodies for the determination of FFA in our study.

Due to the light molecular weight of hapten, it is not able to elicit the immune response of an animal to produce specific antibody. To make it immunogenic, it must be conjugated to a carrier protein (BSA, OVA, etc.) before immunization. Therefore, the hapten must present suitable functional groups (−COOH, −NH_2, −OH, −SH, etc.) to link the molecule to a carrier protein (*28*). Because FFA is a small molecule compound with a free amino group in its structure, the amino group of FFA can be linked to the carrier protein using the cross-linker glutaraldehyde. In this study, immunogen (FFA−BSA) and coating antigen (FFA−OVA) are conjugated by the glutaraldehyde method, and then conjugates FFA−BSA are applied to experimental animals. It was found that the antibodies produced with FFA−BSA conjugates exhibited high affinity toward FFA (**Table 2**) and were more sensitive and specific for FFA (IC_{50} = 3.53 μg/L) than for FF (IC_{50} = 32.44 μg/L). Furthermore, negligible cross-reactivity with other drugs used usually (<0.01%) was obtained (**Table 3**). These results indicated that this linking technique for the preparation of conjugates FFA−BSA by glutaraldehyde is feasible, and the antibodies produced with FFA−BSA are sensitive and specific for FFA.

Working concentrations of antibody and coating antigen were a crucial factor for the sensitivity of ELISA method. In our work, three concentrations of antibody Ab-rabbit$_{10}$ (125, 250, and 375 μg/L) and coating antigen (50, 100, and 200 μg/L) were studied for the selection of the optimum ELISA conditions. As concentrations of antibody Ab-rabbit$_{10}$ gradually decreased, OD values exhibited the same tendency. With the increase of coating antigen, OD values gradually increased (**Figure 4**). It was found from **Table 4** that the coating antigen concentration of 100 ng/mL and antibody Ab-rabbit$_{10}$ concentration of 250 ng/mL were needed to get to the lower IC_{50} values and satisfactory slope and R^2 values. Therefore, for analysis by ELISA, it is necessary to select the optimum concentrations of antibody and coating antigen to improve the sensitivity of the method and get satisfactory OD, slope, and R^2 values.

One of the common challenges of immunoassay for food analysis is matrix interference, which can cause false positives. These matrix interferences could be reduced in a number of ways, such as dilution of sample extract or removal of interferences by sample cleanup procedures using solid-phase extraction or matrix-matched standards. It was not suitable to incorporate the ELISA kits for using solid-phase extraction or the matrix-matched standards. Dilution was a commonly used procedure to reduce the interferences, but this procedure would also reduce the quantifiable sensitivity (*29, 30*). In this study, sample extractions were performed by ethyl acetate/ammonium hydroxide following acid hydrolysis of florfenicol and its known metabolites and were diluted 10-fold prior to analysis by ELISA. Favorable LODs (**Table 5**) and recoveries of FFA (**Table 6**) were obtained. On the basis of the determination of 20 different blank samples, the LODs in swine muscle, chicken muscle, and fish were 3.08, 3.3, and 3.86, respectively, which comfortably satisfied the maximum residue limits (MRLs) in various tissues for FFA residue (**Table 1**). At levels of 5, 50, 100, and 300 μg/kg in the above samples, the recoveries of FFA ranged from 64.6 to 124.7% with low interassay variability (<25.8%). These results indicated that a 10-fold dilution could effectively weaken matrix interference, and this approach worked well with ELISA exhibiting high sensitivity and satisfactory recoveries. However, the concentrations of FFA measured by the ELISA were lower than those by the GC method (**Table 7**). The reason probably was that recoveries of ELISA were lower than those of GC. Therefore, to decrease the risk of false negatives, the sample preparation procedure would be further studied.

Table 5. Determination of 20 Blank Swine, Chicken, and Fish Samples

tissue	concn determined in the ELISA (μg/kg)							av (μg/kg)	SD[a]	LOD[b] (μg/kg)
swine muscle	1.83	1.73	2.52	1.79	1.54	2.33	2.64	2.00	0.36	3.08
	1.92	1.75	1.67	1.96	2.09	2.61	1.76			
	1.87	1.56	1.77	2.16	2.72	1.85				
chicken muscle	2.07	2.76	2.56	1.89	2.31	2.41	2.34	2.28	0.34	3.3
	1.98	1.79	2.11	2.65	2.75	1.87	1.69			
	2.06	2.56	2.73	2.15	2.63	2.28				
fish	2.78	2.65	1.86	1.54	2.87	1.23	1.87	2.15	0.57	3.86
	1.85	2.81	2.09	2.61	1.94	1.49	2.46			
	2.55	1.35	2.86	1.58	2.91	1.76				

[a] SD, standard deviation. [b] LOD, limit of detection = average + 3 SD.

Table 6. Recoveries and Coefficients of the Samples Fortified with FFA in Tissues ($n = 15$)[a]

sample	fortified level (μg/kg)	theor concn in the ELISA[b] (μg/kg)	mean recovery ± SD[c] (%)	interassay variability coefficient (%)
swine muscle	5	0.5	124.7 ± 32.2	25.8
	50	5	82.3 ± 16.1	19.6
	100	10	81.8 ± 9.3	11.3
	300	30	70.5 ± 10.3	14.7
chicken muscle	5	0.5	107.8 ± 21.2	19.7
	50	5	105.3 ± 13.5	12.8
	100	10	84.7 ± 13.0	15.4
	300	30	65.6 ± 13.2	20.1
fish	5	0.5	117.6 ± 26.6	22.6
	50	5	85.9 ± 11.1	12.9
	100	10	78.2 ± 14.5	18.5
	300	30	64.6 ± 10.9	16.9

[a] The test was repeated five times with three replicates per concentration. [b] The extracts were evaporated and diluted by 10-fold with the assay buffer before being analyzed by the ELISA. [c] SD= standard deviation.

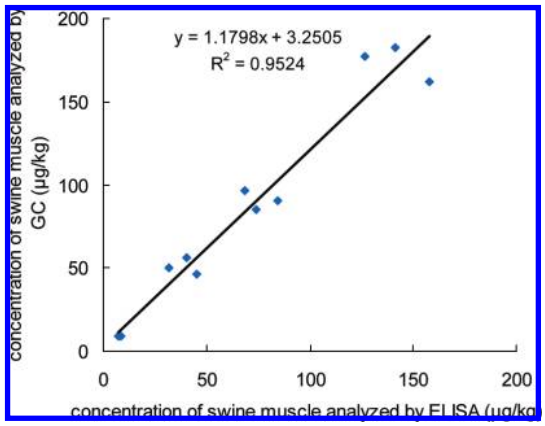

Figure 5. Correlation of FFA assay between the confirmatory GC and the ELISA method in swine muscle samples obtained from FFA spiked at levels of 10, 50, 100, and 200 μg/kg. The regression curve equation was obtained as follows: $y = 1.1798x + 3.2505$, with a correlation coefficient of 0.97.

To assess the capability of determination of the real samples of the ELISA method, the FFA-spiked and field samples were analyzed by the ELISA and GC method. **Figure 5** indicates that an excellent correlation between the ELISA and the reference method was found in the determination of swine muscle ($r = 0.97$). **Table 7** demonstrates that this ELISA could screen the positive samples, which were validated by the reference GC method. The results showed that this ELISA method was reliable for the screening of FFA residue in real edible tissues.

Table 7. Determination of Field Samples Collected from Retail Outlets in Wuhan by the ELISA and GC Methods

tissue	sample	concn determined by ELISA (μg/kg)	concn determined by GC (μg/kg)
swine muscle	3	148.4	181.3
	6	56.2	83.6
	1, 2, 4, 5, 7−10	<LOD	not detected
fish	2	922.3[a]	1203.4
	5	352.5	443.8
	8	104.7	151.3
	1, 3, 4, 6, 7, 9, 10	<LOD	not detected

[a] The samples were diluted 10-fold with the assay buffer before being analyzed by the ELISA, except for sample 2 fish samples with the dilution of 30-fold.

This ELISA is the first reported for FFA detection in animal edible tissues. The LOD of this ELISA method is <4 μg/kg in various tissues based on 20 blank samples accepting no false-positive rates. The recoveries and coefficient of variation of FFA from spiked tissues are also within acceptable range. This method is validated by a good correlation with GC method using spiked samples. The actual results presented in this paper allow this ELISA to be considered as a promising analytical alternative for the quantitative measurements of FFA. In conclusion, a new ELISA method for the detection of FFA following acid hydrolysis in animal edible tissues is established, which has the potential for the development of a rapid test kit.

ABBREVIATIONS USED

FF, florfenicol; FFA, florfenicol amine; TAP, thiamphenicol; CAP, chloramphenicol; CR, cross-reactivity; LOD, limit of detection; BSA, bovine serum albumin; OVA, ovalbumin; DMF, *N*,*N*-dimethylformamide; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; ic-ELISA, indirect competitive enzyme-linked immunosorbent assay; TMB, 3,3′,5,5′-tetramethylbenzidine; μECD, microcell electron capture detector; CV, coefficient of variation; SD, standard deviation; IC_{50}, 50% inhibition values.

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