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Development of an Indirect Competitive ELISA for Flumequine Residues in Raw Milk Using Chicken Egg Yolk Antibodies

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To detect flumequine in raw milk, an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed. By carbodiimide conjugation, flumequine was conjugated to cationized bovine serum albumin (cBSA−flumequine) and to cationized ovalbumin (cOVA−flumequine). For the immunization of chickens, cBSA−flumequine was used, which allowed the isolation of specific chicken egg yolk immunoglobulins (IgY) for flumequine. As the coating antigen in the immunoassay, cOVA−flumequine was used. In the indirect competitive assay, standard flumequine was incubated together with the anti-flumequine antibodies. The antibody by which the lowest concentration of free flumequine that gives 50% inhibition of binding (IC_{50}) was found in aqueous dilution was further tested for the applicability to detect flumequine in raw milk. An IC_{50} level in milk was reached that was about 5 times lower than in aqueous solution. So flumequine can be detected directly in raw milk at maximum residue level (50 μg/kg). No cross-reactivity was noticed with various related quinolones.

KEYWORDS: Flumequine; fluoroquinolones; milk; IgY; chicken immunoglobulins; ELISA

INTRODUCTION

Residues of antibiotics in milk are a risk for further processing (e.g., inhibition of fermentation) and also for the consumer (e.g., toxicity, allergy, antibiotic resistance). For these reasons, the use of antibiotics in animal production is strictly regulated and maximum residue levels (MRLs) have been established for countries of the European Union (*1*).

Fluoroquinolones are synthetic antibiotics with a mode of action based on selective inhibition of bacterial DNA synthesis by targeting the essential bacterial enzymes DNA gyrase and topoisomerase IV (*2*). At present, this group of antibiotics is widely used in animal production (*3*). European Union MRLs in milk have been set for enrofloxacin (+ciprofloxacin) (100 μg/kg), flumequine (50 μg/kg), marbofloxacin (75 μg/kg), and danofloxacin (30 μg/kg) (03/2004) by Council Regulation EEC/2377/90 (and amendments). To test samples for the presence of these substances, rapid and inexpensive screening methods are required. The microbiological inhibitor assay used for screening of quinolones in milk only detects enrofloxacin (+ciprofloxacin) at the MRL (*4*). Until recently, commercial immunoassays available for screening of fluoroquinolone residues were restricted to enrofloxacin and ciprofloxacin. Furthermore, immunological methods described in the literature for detection of fluoroquinolones are also limited (*5*−*8*) and to our knowledge, no immunochemical assay for detection of flumequine in milk has been described.

Despite the fact that chickens are very good producers of antibodies, the use of chicken immunoglobulins in immunochemical assays is currently limited in comparison to mammalian antibodies. However, a recently published paper demonstrates the usefulness of chicken egg yolk immunoglobulins (IgY) for the development of an ELISA for the small chemical compound bisphenol A (*9*).

In the present paper, the use of chicken immunoglobulins in the development of an ELISA for detection of flumequine in raw milk at the MRL is described.

MATERIALS AND METHODS

Reagents and Buffers. 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethylenediamine dihydrocloride (EDA), flumequine, oxolinic acid, sodium phosphate dibasic, bovine serum albumin (BSA), ovalbumin (OVA), tetramethylbenzidine dihydrochloride (TMB), hydrogen peroxide (30%), Freund's adjuvant incomplete (FIA), Freund's adjuvant complete (FCA), rabbit anti-chicken IgG-peroxidase conjugate, and Antifoam A were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxysulfosuccinimide (sulfo-NHS) was purchased from Pierce (Rockford, IL). Proclin-300 was from Zymed Laboratories, Inc. (San Francisco, CA). Enrofloxacin and ciprofloxacin were from Bayer (Kansas City, MO), difloxacin was supplied by Fort Dodge Animal Health (Overland Park, KS), marbofloxacin was provided by Vétoquinol (Lure cedex, France), and danofloxacin was from Pfizer Inc. (Groton, CT). All other chemicals used were chemical grade from Sigma-Aldrich.

For the preparation of all buffers and reagents for the immunoassays, ultrapure Milli Q water was used and 0.05% Proclin-300 was added as a preservative. Phosphate-buffered saline (PBS) (pH 7.4) consisted of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl.

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Table 1. Immunization Scheme of the Laying Hens

injection	point of time (day)	Δt (days)	cBSA–flumequine (μg)	adjuvant/buffer
primary	0	0	500	FCA/PBS (1/1)
booster 1	21	21	250	FIA/PBS (1/1)
booster 2	35	14	250	–/PBS
booster 3	49	14	125	–/PBS
booster 4	77	28	125	–/PBS
booster 5	105	28	125	FIA/PBS (1/1)

As a coating buffer, 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$; pH 9.6) was used. The wash buffer consisted of PBS + 0.05% (v/v) Tween-20 + 0.004% Antifoam A. The blocking solution was PBS with 2% Na-caseinate. The assay buffer was PBS + 0.05% (v/v) Tween-20 + 0.1% Na-caseinate. The substrate buffer was 0.1 M sodium acetate/citrate buffer pH 5.0. To prepare the substrate solution, 6 mg of TMB was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and 332 μL of this solution was added to 10 mL of substrate buffer plus 3 μL of H_2O_2 (30%). The stopping solution was 1 M H_2SO_4.

To prepare skim milk from raw milk, raw milk was centrifuged for 10 min at 1800*g* and subsequently held for 30 min at 4 °C to solidify the fat. Subsequently, the liquid skim milk fraction was isolated from the solid fat fraction.

Preparation of Flumequine Conjugates. The immunogen cBSA−flumequine and the coating antigen cOVA−flumequine were prepared by carbodiimide modification, based on described methods (*10*). In a first step, carboxylic acids of the carrier proteins BSA and OVA were converted to primary amines with an excess of EDA. BSA or OVA was added in a concentration of 5 mg/mL to a solution containing 1 M EDA in 0.1 M sodium phosphate buffer (pH 7.4). To this solution, EDC was added to a final concentration of 2 mg/mL, followed by incubation at room temperature for 2 h. Subsequently, the solution was dialyzed (molecular weight cutoff (mwco), 12 000−14 000) against 0.1 M sodium phosphate buffer (pH 7.4). Cationized BSA and OVA carrier proteins were defined as cBSA and cOVA. In a second step, flumequine was coupled to cBSA and cOVA through its carboxyl group. To each carrier (5 mg/mL in 0.1 M sodium phosphate buffer (pH 7.4)), 15 mM flumequine was added. To this solution, EDC and sulfo-NHS were added to final concentrations of 0.1 M and 25 mM, respectively. The solution was incubated for 2 h at room temperature, followed by dialysis (mwco, 12 000−14 000) against PBS.

Determination of the efficiency of conjugation of flumequine to the carrier molecule was carried out by UV spectroscopy at 330 nm. At this wavelength, flumequine absorbs maximally while the carrier protein does not absorb at all. A calibration curve was made for flumequine, by plotting the absorption at 330 nm of flumequine solutions with different known concentrations against the concentration. By means of extrapolation, the flumequine concentration in the flumequine−carrier conjugate was determined. By taking into account the protein carrier concentration and the molecular weights of both flumequine and carrier, the molar ratio of flumequine/carrier was calculated.

Immunization of the Chickens and Isolation of the IgY Antibodies. Three Isa Brown chickens of 30 weeks old were injected intramuscularly at different times with 500 μL of the immunogen (cBSA−flumequine) in PBS with or without adjuvant (**Table 1**). Daily, the eggs were collected from each individual chicken. IgY antibodies were isolated from the egg yolk using the method described by Akita and Nakai (*11*), with minor modifications. Briefly, the egg yolk was separated from the egg white and diluted (1:10) with distilled water. The pH was set with 1 N HCl between 5.0 and 5.2. After overnight incubation at 4 °C, the mixture was centrifuged (10000*g*, 1 h, 4 °C) and the supernatant was filtered through a Whatman filter paper (retention of 20−25 μm particles). Subsequently, 170 mL of distilled water and 72 g of ammonium sulfate were added. This solution (about 60% saturated solution of ammonium sulfate) was incubated for 1 h at room temperature (RT) and then centrifuged for 20 min (10000*g*, RT). The pellet was dissolved in 19% (w/v) sodium sulfate and incubated for 20 min at RT. After centrifugation (2000*g*, 20 min, RT), the pellet was dissolved in 14% (w/v) sodium sulfate, incubated for 20 min, and centrifuged for 20 min (2000*g*, RT). The final pellet was dissolved in PBS + 0.02% sodium azide and stored in aliquots at −20 °C. Working stock solutions of the IgY preparations of 1/20 in PBS + glycerol (1/1) were made and stored at −20 °C. These were further diluted before use.

Antibody Titer Determination by Indirect ELISA. For all ELISA assays, Microlon 600 microtiterplates (Greiner Bio-One NV/SA, Wemmel, Belgium) were used. The titers of the different IgY antibody preparations were tested by indirect ELISA, using the protocol described below. Wells of the microtiterplates were coated with the coating antigen cOVA−flumequine at 4 μg/mL (50 μL/well) by overnight incubation at 4 °C. Plates were washed (3×) with wash buffer and blocked with 200 μL of blocking solution per well for 1 h at 37 °C. Plates were washed again (3×), the appropriate dilution of the IgY preparation (primary antibody; diluted from the working stock with assay buffer) was added, and the plates were incubated for 2 h at 37 °C while shaking (250 rpm). Plates were washed (5×), and anti-chicken IgY-HRP (1/10 000; 50 μL/well) was added followed by an additional incubation for 1 h at 37 °C while shaking (250 rpm). Next, the plates were washed (5×), TMB substrate solution was added (50 μL/well), and the plates were additionally incubated for 30 min at 37 °C. Finally, 50 μL of stopping solution was added and the absorbance was measured at 450 nm. Absorbances were corrected for blank readings (wells in which no primary antibody was added). IgY preparations from eggs of preimmune chickens were included as a negative control, and the antibody titer was defined as the IgY dilution factor that gives an absorbance of 0.1 above that of the preimmune chickens.

Development of the Indirect Competitive ELISA. Checkerboard tests were performed to determine optimal dilution of the coating antigen and the primary and secondary antibody for the indirect competitive ELISA, resulting in the following optimized protocol: wells were coated with cOVA−flumequine at 100 ng/mL (100 μL/well) by incubation overnight at 4 °C, followed by washing (3×). Plates were blocked (200 μL/well) for 1 h at 37 °C and washed again (3×). For the competition step, 70 μL of the appropriate flumequine dilution (in assay buffer or in raw milk) and 30 μL of the primary antibody solution were added to each well. The final dilutions of the primary antibodies C1-114 and C3-112 in the competition step were 1/4000 and 1/16 000, respectively. Plates were incubated for 2 h at 37 °C with shaking (250 rpm). Subsequently, the plates were washed (5×), 100 μL of anti-chicken IgY-HRP (1/40 000 in assay buffer) was added, and the plates were incubated for 1 h at 37 °C while shaking (250 rpm). Next, the plates were washed (5×) and the substrate solution (100 μL/well) was added, followed by incubation for 30 min at 37 °C. Finally, 100 μL of stopping solution was added and the absorbance was measured at 450 nm. The detection limit is defined as the lowest concentration of flumequine that exhibits a signal which is lower than the signal of the negative sample (absorbance value in the absence of analyte B_0) diminished by 3 times the standard deviation (B_0 − 3SD). The IC_{50} is defined as the concentration of the compound that gives half of the maximum signal intensity. Standard curves were normalized by expressing experimental absorbance values (B) as $B/B_0 \times 100$.

Specificity of the Assay. Competitive immunoassays (in assay buffer and in raw milk) were performed using various compounds structurally related to flumequine, to determine the respective IC_{50} value and cross-reactivity. The tested compounds were enrofloxacin, ciprofloxacin, difloxacin, danofloxacin, marbofloxacin, and oxolinic acid. The used ELISA procedure was similar to the optimized ELISA protocol (described above), except for the competition step. Here, 70 μL of the appropriate compound (at a concentration ranging from 100 μg/kg to 100 mg/kg) diluted in assay buffer or in raw milk and 30 μL of the primary antibody C3-112 (1/4800 diluted in assay buffer, resulting in a final dilution of 1/16000 in the competition step) were added to the wells. The cross-reactivity (%) was calculated as $(IC_{50,flumequine})/(IC_{50,compound}) \times 100$.

RESULTS AND DISCUSSION

Preparation of Flumequine Conjugates. Flumequine is a small molecule with a molecular weight of 261.3. To prepare the immunogen and the coating antigen, this molecule was

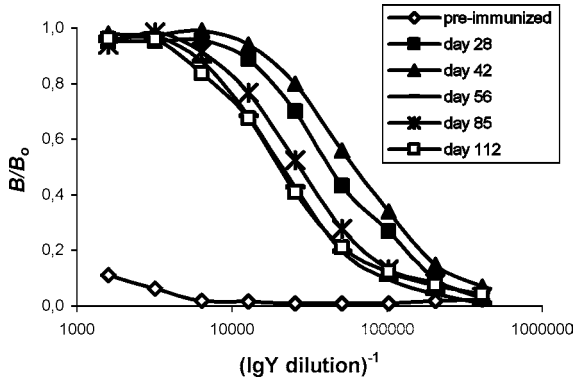

Figure 1. Response of a chicken immunized with the cBSA–flumequine conjugate at various days after start of the immunization. The response was normalized to the maximum absorbance measured, B_0.

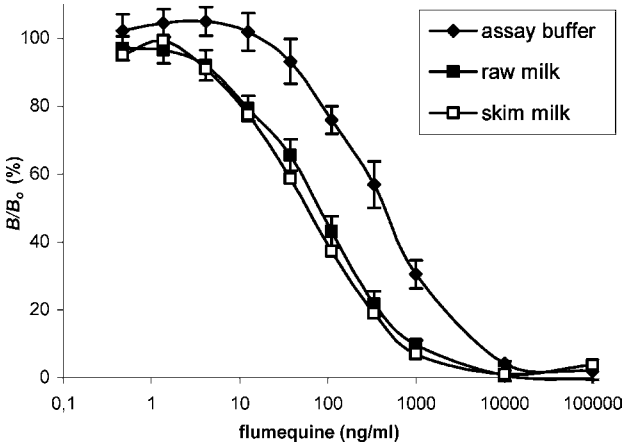

Figure 2. Indirect competitive ELISA standard curves for flumequine in aqueous dilution (assay buffer), in raw milk, and in skim milk, using the polyclonal chicken antibody C3-112 (1/16 000).

conjugated to protein carrier molecules. BSA and OVA were used as carriers. These were first modified with the diamine EDA to prepare the cationized carriers cBSA and cOVA. In a second step, flumequine was coupled by its carboxyl group to the amino groups of the cationized carriers, resulting in the immunogen (cBSA−flumequine) and the coating antigen (cBSA−ovalbumine). Coupling efficiencies were evaluated by UV spectroscopy and revealed 13.91 and 8.08 mol flumequine per mol cBSA and cOVA, respectively. The use of cationized carriers has the advantage that more amino groups on the carrier become available for coupling and that protein cross-linking is minimized. Furthermore, cationized proteins modified with diamines to increase their pI are known to generate an increased immune response compared to their native forms (*10*).

Antibody Titer Determination. As the immunogen in the different immunization steps, cBSA−flumequine was used (**Table 1**). After each immunization, eggs were collected and IgY antibodies were isolated. The immune response of the different animals was checked by an indirect ELISA. All three immunized hens reacted clearly toward the administered immunogen. In **Figure 1**, the immune response of one chicken (chicken 3) is visualized. After the first booster immunization (day 28), a titer of <100 000 was generated. A second booster injection resulted in a slight augmentation of the titer. After the third and fourth immunization, a decline of the titer was recorded. A fifth booster reaction was performed with addition of FIA (**Table 1**), but this did not result in an augmentation of the titer. A similar immune response profile, this is a high titer after the first booster immunization but no additional augmentation of the titer after additional booster injections, was found by De Meulenaer et al. (*9*), who made use of chickens for production of antibodies against the small organic molecule bisphenol A. It is not clear whether this is due to the use of chickens. At present, the use of chickens for production of polyclonal antibodies against small organic compounds is very limited. Based on our literature survey, this is the first report of use of chickens for production of antibodies that recognize an antibiotic molecule.

Development of the Indirect Competitive ELISA. From the collection of isolated IgY antibodies, 10 preparations showing the highest titers were selected. These were evaluated in an indirect competitive ELISA for their potency to detect free flumequine in aqueous dilution (assay buffer). In the indirect competitive ELISA, the signal (optical density) is inversely proportional to the concentration of flumequine in the samples. Two antibodies (C1-114 (from chicken 1, day 114) and C3-112 (from chicken 3, day 112)) were clearly more potent than the others for the detection of free flumequine in solution, as a lower signal (optical density) at the same flumequine concentration in the samples was obtained with these antibodies. Both preparations were used to further optimize the competitive ELISAs. Several assay parameters were optimized by checkerboard analysis. Of these, the concentration of the coating antigen was found to have the highest impact on the sensitivity of the assay. The optimal coating concentration was found to be 100 ng/mL. The optimal concentrations of the primary antibodies C1-114 and C3-112 were 1/4000 and 1/16 000, respectively. The optimal secondary antibody concentration (anti-chicken IgY-HRP) was 1/40 000. The concentration of flumequine that gives half of the maximum signal intensity (IC_{50}) was determined for C1-114 and C3-112. IC_{50} values of about 500 μg/kg were reached in aqueous dilution (assay buffer). However, with the C3-112 antibody a standard curve with a better displacement of free flumequine was obtained. In **Figure 2**, the flumequine standard curves obtained with C3-112 are given. As an MRL (50 μg/kg) has been set for flumequine in raw milk, the performance of the developed assay was evaluated for raw milk. Without an extraction step, an IC_{50} value of 90 μg/kg was obtained, which is about 5 times lower than in assay buffer. This means that about 5 times less free flumequine is necessary to inhibit 50% of the IgY binding to the coating antigen cOVA−flumequine in raw milk than in assay buffer, resulting in a more sensitive assay and thus a lower detection limit. The detection limit of flumequine in raw milk is about 12.5 μg/kg, making it possible to detect flumequine at the MRL in this matrix. To evaluate further the performance of the assay, 20 different blank raw milk samples were spiked with 12.5 μg/kg flumequine. The average B/B_0 (%) detected was 73.7 $\pm$ 6.1, indicating that the variability of the composition of raw milk does not affect the detection limit. The low IC_{50} value obtained in raw milk in comparison to the IC_{50} in assay buffer is rather unexpected, as others found an increased IC_{50} value when milk was used instead of aqueous dilution (*9*). To test the influence of the fat content of raw milk on the competition curve, the lipid fraction of the raw milk was removed by centrifugation and the prepared skim milk was analyzed (**Figure 2**). The flumequine competition curve in skim milk is similar to that in raw milk, indicating that the fat content of the raw milk is not responsible for the higher sensitivity of the assay.

Specificity of the Assay. The specificity of the developed assay was evaluated by determination of the cross-reactivity toward various structurally related quinolones (e.g., enrofloxacin, ciprofloxacin, difloxacin, danofloxacin, marbofloxacin, oxolinic acid). This was performed by an indirect competitive ELISA

in which free competitors were added at different concentrations (ranging from 100 μg/mL to 100 mg/mL) and evaluated for their potency to compete with the binding of the C3-112 antibody to the coating antigen. All the tested quinolones showed a cross-reactivity of less than 0.1% in assay buffer as well as in raw milk, indicating that the developed immunoassay had a high specificity for flumequine. Immunoassays developed against sarafloxacin and ciprofloxacin showed cross-reactivities between 1 and more than 100% with related fluoroquinolones (*5*,*6*). The high specificity observed is probably due to the small size of flumequine and the different structure in comparison with the other tested fluoroquinolones.

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