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Biosensor immunoassay for flumequine in broiler serum and muscle

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Abstract

Flumequine (Flu) is one of the fluoroquinolones most frequently applied for the treatment of broilers in The Netherlands. For the detection of residues of Flu in blood serum of broilers, a biosensor immunoassay (BIA) was developed which was fast (7.5 min per sample) and specific (no cross-reactivity with other (fluoro)quinolones). This inhibition assay was based on a rabbit polyclonal anti-Flu serum and a CM5 biosensor chip coated with Flu which could be detected in the range of 15–800 ng mL^{-1}.

For the detection of Flu in muscle, an easy extraction procedure in buffer was selected and the measuring range was from 24 to 4000 ng g^{-1}. Average recoveries of 66 till 75% were found with muscle samples spiked at 0.5, 1 and 2 times the maximum residue limit (MRL in muscle = 400 ng g^{-1}) and the decision limit (CCα) and the detection capability (CCβ) were determined as 500 and 600 ng g^{-1}, respectively.

Incurred muscle samples were analysed by the BIA and by LC-MS/MS and a good correlation was found ($R^2 = 0.998$). Serum and muscle samples from with Flu treated broilers were analysed and the concentrations found in serum were always higher than those found in muscle (average serum/muscle ratio was 3.5) and this proved the applicability of the BIA in serum as predictor of the Flu concentration in muscle.

Keywords: Biosensor immunoassay; Surface plasmon resonance; Flumequine; Broiler; Serum; Muscle

1. Introduction

"The development of chain oriented monitoring and surveillance in the poultry chain" was the title of a Dutch research project in which biosensor options for the on-site and simultaneous detection of pathogens and drug residues in broiler slaughterhouses were evaluated. Automated optical biosensors of the company Biacore AB were chosen and, to perform combined assays, the Biacore 3000, with four serially connectable flow channels (Fcs), was selected. Blood serum of broilers was chosen as the matrix for the detection of antibodies against pathogens (i.e. *Salmonella*) and drug residues with sulfonamides as the first model compounds. For the biosensor detection of sulfonamides in broiler serum, different approaches, ranging from specific to multi-sulfonamide assays, were followed [1]. In the finally selected multi-sulfonamide biosensor immunoassay (BIA), a mutant antibody (M.3.4) was used in combination with a sulfonamide-derivative coated on the surface of the biosensor chip in one of the flow channels (Fcs) [2]. The sample preparation consisted of a dilution in buffer only and with this BIA, all 17 sulfonamides tested, including the five sulfonamides registered for application in broilers, could be detected in broiler serum with limits of detection between 4 and 82 ng mL^{-1}. For the application in a combined assay with the detection of anti-salmonella, the biosensor conditions (sample dilution, sample buffer, running buffer and regeneration conditions) were adapted to the serological assay conditions (using lipopolysaccharides (LPS)-coated chips [3]). The adapted BIA was successfully applied during the analysis of samples from an animal experiment and during a survey in which 310 broiler serum samples of 31 different flocks were tested for anti-salmonella and sulfonamides [4].

However, other antibiotics can be used and for instance the quinolones are frequently applied veterinary drugs of which flumequine (Flu) is the most applied in broilers in The Netherlands. To establish safe limits for human consumption, the European Union (EU) established maximum residue limits (MRL) for Flu in chicken edible tissues of 1000, 800, 400 and 250 ng g^{-1} in kidney, liver, muscle and skin + fat, respectively [5].

For the development of a BIA, antibodies are necessary and different approaches for raising antibodies against

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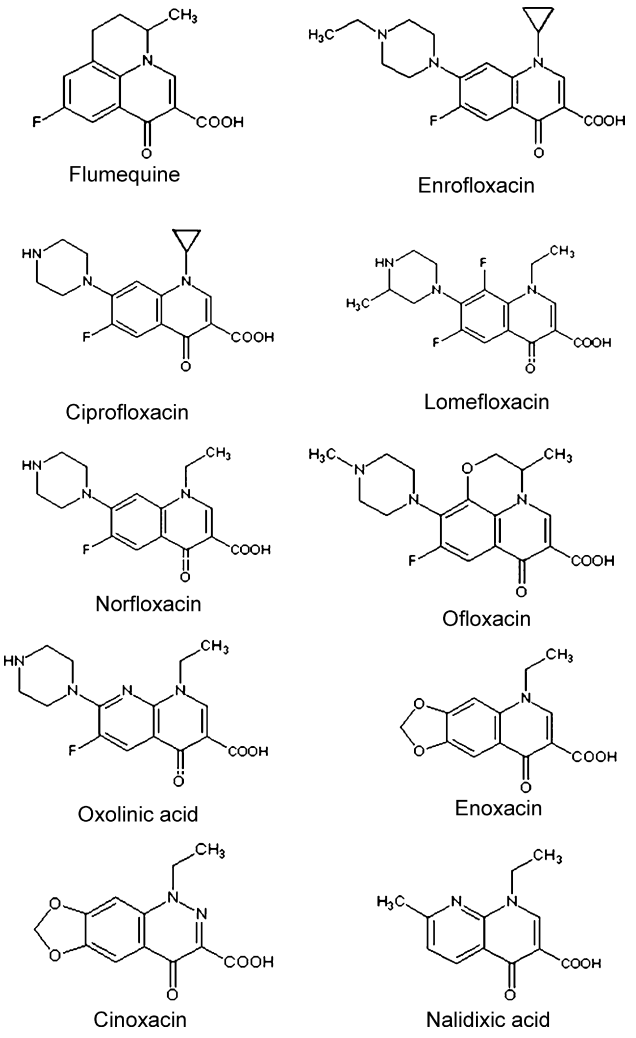

Fig. 1. Structures of (fluoro)quinolones.

(fluoro)quinolones were described previously [6–13]. Polyclonal [6,7] and monoclonal antibodies [8] were developed against sarafloxacin and cross-reactivities (CR's) were observed with structurally related quinolones including difloxacin, enrofloxacin, norfloxacin, trovafloxacin and nalidixic acid. Monoclonal antibodies against enrofloxacin were highly specific [9] whilst polyclonal antibodies against enrofloxacin [10] showed CR with ciprofloxacin and antibodies against ciprofloxacin showed CR with enrofloxacin and norfloxacin [11]. An ELISA with polyclonal antibodies raised against norfloxacin, linked to ovalbumin via the secondary amine group as found in the piperazine moiety of norfloxacin, was able to detect nine different (fluoro)quinolones [12]. However, the CR towards Flu was 6% only. In the same study, highly specific ELISA's were obtained with polyclonal antibodies against ciprofloxacin, enrofloxacin, Flu and nalidixic acid. The Flu ELISA in milk showed 50% inhibition at 29 $\mu g\,kg^{-1}$ and that is three to four times more sensitive than obtained with highly specific chicken egg yolk antibodies against Flu [13]. As shown in Fig. 1, Flu has a deviating structure compared to the other quinolones. This explains the high specificity of the antisera against Flu.

In the present study, because of the availability, antisera against Flu were raised in rabbits using two immunogens (Flu-bovine serum albumin (BSA) and Flu-keyhole limpet hemocyanin (KLH)). The antisera were tested in an ELISA and in the Biacore using a CM5 biosensor chip coated with Flu in which a previously described two-step immobilization procedure [10], with ethylene diamine as spacer, was applied. For the detection of Flu in broiler sera, the easy sample preparation (dilution in anti-Flu containing buffer only) was adapted to the conditions previously described for the detection of anti-salmonella and sulfonamides [4]. Maximum residue limits (MRLs) for serum are not prescribed and the MRL in muscle of 400 $ng\,g^{-1}$ was chosen as the minimum required performance limit (MRPL) in serum and, under these conditions, the decision limit (CCα) and the detection capability (CCβ) for Flu in serum were determined. The BIA was further applied during a survey with 310 broiler serum samples obtained from 31 different farms and sera from treated and untreated broilers were analysed. For the application of the BIA in muscle, a simple extraction procedure was used, and CCα and CCβ were determined using blank muscle samples analysed with and without the addition of Flu at the MRL level. The BIA results were compared with LC-MS/MS results using incurred samples. Serum and muscle samples obtained from broilers treated with Flu were analysed and the ratio of Flu in both materials was determined to evaluate the applicability of serum levels as predictor for Flu levels in muscle.

2. Materials and methods

2.1. Materials

Piromidic acid was supplied by ICN Biochemicals (Ohio, USA) and marbofloxacin by Laboratoire Pharmaceutique Veterinaire (Lure Cedex, France). Enrofloxacin, ciprofloxacin and CM-dextran sodium salt were obtained from Fluka Chemie (Zwijndrecht, The Netherlands) and difloxacin from Abbott Laboratories (North Chicago, IL, USA). Isobutyl chloroformate, Tween-20, Tween-80, *N*,*N*-dimethylformamid (DMF), tributylamine (TBA), dimethylsulfoxide (DMSO), ethylene diamine (EDA) and acetonitrile were obtained from VWR International (Amsterdam, The Netherlands). EIA grade horseradish peroxidase (HRP) was from Roche Diagnostics (Mannheim, Germany). HBS-EP buffer (containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% Surfactant P20), CM5 biosensor chips and the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride–NaOH (pH 8.5)) were supplied by Biacore AB (Uppsala, Sweden). Goat anti-rabbit IgG was obtained from Caltag Laboratories (Burlingame, CA). COSTAR® ELISA microtitre plates were obtained from Corning Incorporated (Corning, NY 14831, USA). Solutions of tetramethylbenzidine (TMB) peroxidase substrate and peroxidase were obtained from Kirkegaard and Perry Labs (Gaithersburg, MD, USA). Flumequine (Flu), ofloxacin, enoxacin, cinoxacin, oxolinic acid, norfloxacin, naldixic acid, lomefloxacin, bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH),