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Dual biosensor immunoassay-directed identification of fluoroquinolones in chicken muscle by liquid chromatography electrospray time-of-flight mass spectrometry

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Abstract

Fluoroquinolones (FQs) are synthetic antibiotics of broad-spectrum antibacterial activity widely used to treat infections in farmed fish, turkeys, pigs, calves and poultry. Monitoring these substances residues is therefore regulated by law.

For the detection of FQs, we studied the feasibility of coupling the simultaneous screening of several FQs, using a dual surface plasmon resonance (SPR) biosensor immunoassay (BIA), in parallel, with an analytical chemical methodology for their identification.

Six FQs were simultaneously screened at or below their maximum residue level (MRL) in chicken muscle using a multi-FQ BIA for norfloxacin, ciprofloxacin, enrofloxacin, difloxacin and sarafloxacin, and a specific BIA for flumequine. The two BIAs were serially coupled in a multi-channel SPR biosensor featuring a dual BIA in a competitive inhibition format.

The samples non-compliant during the screening with the dual BIA were further concentrated and fractionated with gradient liquid chromatography (LC). The effluent was splitted toward two 96-well fraction collectors resulting in two identical 96-well plates. One was re-screened with the dual BIA to identify the immunoactive fractions and direct the identification efforts toward the relevant fractions in the second well-plate with high resolution LC-electrospray time-of-flight mass spectrometry (ESI-TOFMS). The system not only allows the possibility to screen and identify known FQs, but also to discover unknown chemicals of similar structure which show activity in the dual BIA.

Keywords: Fluoroquinolones; Biosensor immunoassays; Generic antibodies; Surface plasmon resonance; Time of flight mass spectrometry; High resolution liquid chromatography

1. Introduction

Profitability of livestock is often hampered by infectious diseases, therefore the use of veterinary antibacterial drugs is expected for treatment and prophylaxis. Fluoroquinolones (FQ) belong to a new generation of broad-spectrum antibiotics capable of inducing bacterial death by inhibiting DNA gyrase [1]. FQs are active against Gram negative and Gram positive bacteria [2] and are widely used to treat infections, as prophylactic and growth promoters in farmed fish, turkeys, pigs, calves and poultry [3]. Therefore, raising public health concerns relating to allergic reactions and the generation of antibiotic resistant bacterial strains [4,5]. In consequence, a national veterinary residue monitoring plan for these and other groups of substances should be established by the EU member countries in accordance to EC Council Directive 96/23/EC [6].

Consequently, there is a rising need for rapid, robust, multi residue screening assays coupled to identification methods for these low molecular weight compounds.

Several methods have been described for the determination of quinolone residues using liquid chromatography (LC) with fluorescence detection [1,7,8], with Ultra Violet (UV) detection

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[1,9,10] and LC-mass spectrometry (MS) [11]. Alternative methods like a microbiological assay [12,13], specific and multi-FQ immunoassays have also been reported [14–17].

Within the scope of the EU project BioCop (www.biocop.org) several biosensor-based screening methods are being developed for chemical contaminants in food. Our challenge within this project is to evaluate strategies for coupling the biosensor screening methods with MS for confirmation of identity and/or the identification of unknowns having activity in the biosensor assays. In the present study we coupled a dual inhibition biosensor immunoassay (BIA) in a multi-channel surface plasmon resonance (SPR) biosensor for the screening of FQs in chicken muscle samples in parallel with high resolution LC/MS identification. This concept was previously evaluated using yeast bioassay-directed identification of estrogen and androgen residues in urine [18,19].

In the dual BIA for the FQs, a multi-FQ BIA was developed for the detection of norfloxacin (Nor), ciprofloxacin (Cipro), enrofloxacin (Enro), difloxacin (Diflo) and sarafloxacin (Sara) was coupled to a specific BIA for flumequine (Flu).

Samples considered positive during the screening with the dual BIA were concentrated with solid phase extraction (SPE) and separated with a standard HPLC of which the effluent was splitted toward two identical 96-well fraction collectors. The fractions in the 96-well plate were re-screened for immunoactivity using the dual BIA, this generated an immunoaffity chromatogram or immunogram [20]. In the immunogram the immunoactive fractions positions were clearly distinguished and the immunoactive wells positions were used in the second 96-well plate for immunoactive oriented identification using high resolution HPLC, electrospray ionization (ESI) time-of-flight mass spectrometry (TOFMS).

2. Experimental

2.1. Materials

Sensor chips (CM5), HBS-EP buffer [pH 7.4, consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant polysorbate (P20)] and an 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). Ethylene diamine (EDA) was obtained from VWR International (Amsterdam, The Netherlands). ELISA microtitre plates were supplied by Greiner (Frickenhausen, Germany).

Polyclonal antisera were raised in rabbits against a Flu conjugate (PAb MH41) [16] and a Nor-COOH derivative (see Fig. 2) conjugated to a carrier protein (PAb CA65) [17,21]. Acetonitrile HPLC far-UV grade was from Lab-Scan Ltd. (Dublin, Ireland). Water was purified using a Milli-Q gradient A10 system (Millipore, Bedford, MA). Solid Phase Extraction (SPE) (20 mg *N*-vinylpyrrolidone/divinylbenzene based sorbent) OASIS HLB cartridges were from Waters (Milford, MA, USA). The 0.45 μm Durapore and 30 kDa cutoff Amicon filters were from Millipore (Amsterdam, The Netherlands). Cipro and Enro were from Fluka Chemie (Zwijndrecht, The Netherlands). Diflo was from Abbott Laboratories (North Chicago, Illinois, USA). Sara was from Dr. Ehrenstorfer (Augsburg, Germany). Nor, Flu, 5-aminovaleric acid (AVA) and all other reagents where obtained from Sigma–Aldrich Chemie BV (Zwijndrecht, The Netherlands) unless otherwise stated.

2.2. Solutions and samples

The FQs mixture (FQs mix) was prepared using standards of Nor, Cipro, Enro, Diflo, Sara and Flu in methanol (10 mg L^{-1}). Muscle samples from blank broilers and broilers treated with Enro were obtained from an animal experiment performed at the Animal Sciences Group (ASG; Lelystad, The Netherlands). The spiked blank chicken muscle samples were prepared by adding 100 μL of a FQs mix (1 mg L^{-1}) to 1 g of chicken muscle, achieving a level of 100 ng g^{-1} for each of the FQs before extraction. To prepare the incurred chicken muscle samples, chickens were dosed with Enro at 30 mg kg^{-1} total body weight through the drinking water for 5 consecutive days. This resulted in 2700–6800 μg kg^{-1} levels, far above the maximum residue levels (MRLs), hence the samples were diluted with blank chicken samples until a level of 0.5 MRL (50 μg kg^{-1}) was reached. The official Dutch committee for ethics on animal experiments (DEC) approved these animal experiments.

2.3. Equipment

The experimental system setup (Fig. 1) consisted of a dual SPR-based inhibition BIA, a gradient LC, an autosampler, a dual 96-well fraction collector and a high resolution LC-QTOFMS. The BIACORE 3000 was supplied by Biacore AB (Uppsala, Sweden). The gradient LC system consisted of two Knauer (Berlin, Germany) model WellChrom K-1001 pumps, a Knauer high pressure dynamic mixing chamber, a GasTorr model 154 membrane degasser, and an autosampler model Endurance from Spark Holland (Emmen, The Netherlands). Liquid chromatography was performed using a Waters (Milford, MA) 150 mm × 3 mm i.d. Symmetry column packed with 5 μm C18 material. The column effluent was split toward two identical Gilson (Villiers-le-Bel, France) model FC203B 96-well fraction collectors.

Identification was performed using a Waters Acquity UPLC system equipped with a 50 mm × 2.1 mm i.d. column packed with 1.7-μm BEH C18 material. The LC column was directly interfaced with a Micromass (Manchester, UK) model QTOF micro MS system.

2.4. Procedures

2.4.1. Sample preparation and solid phase extraction

Chicken muscle samples were extracted by homogenizing 1 g of chicken breast tissue with 10 mL of water. The homogenate was centrifuged and the supernatant was filtered through a 0.45 μm filter. Two milliliters of the filtrate were ultrafiltrated