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Analytical Methods

Immunochemical determination of fluoroquinolone antibiotics in cattle hair: A strategy to ensure food safety

Fátima Fernández [a,b], Daniel G. Pinacho [a,b], Marta Gratacós-Cubarsí [c], José-Antonio García-Regueiro [c], Massimo Castellari [c], Francisco Sánchez-Baeza [a,b,1], M.-Pilar Marco [a,b,*]

[a] Nanobiotechnology for Diagnostics group (Nb4D), IQAC-CSIC, Jorge Girona, 18-26, 08034 Barcelona, Spain
[b] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Jorge Girona, 18-26, 08034 Barcelona, Spain
[c] Institute for Food and Agricultural Research and Technology, Meat Technology, Centre (IRTA-CTC) Food Chemistry Unit, Granja Camps i Armet s/n, Monells, 17121 Girona, Spain

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ABSTRACT

Enrofloxacin (ERFX) is a synthetic antibiotic of the fluoroquinolone (FQ) family, which is commonly administered in veterinary medicine. ERFX and its metabolite, ciprofloxacin (CPFX), have been reported to accumulate in hair of treated animals. Therefore, hair analysis is an attractive non-invasive alternative to control misuse of such antibiotic and to ensure food safety by preventing such food derived products arrive to the consumer. In this context, an immunochemical analytical protocol has been established to detect ERFX and CPFX residues in cattle hair samples. Unpigmented and pigmented hair were collected from ERFX-treated and non-treated calves, and the aqueous NH_4OH extracts were directly analyzed by ELISA, being possible to achieve limits of detection in the range of 10–30 μg kg^{-1}. A good concordance between HPLC and ELISA measurements was observed. The results demonstrate the potential of the immunochemical procedure reported here to rapidly screen and quantitate FQ residues in hair samples.

1. Introduction

Quinolones are a group of synthetic antibiotics employed in veterinary and human medicine for the treatment of urinary, pulmonary and digestive infections, from which ones fluoroquinones (FQ) constitute an important group. Several generations of quinolones have been developed since 1982 to improve their efficiency against different bacteria (Oliphant & Green, 2002). Unfortunately, as other antibiotics, quinolones have been misused as growth promoters. This abuse has greatly contributed to the development of antimicrobial resistance, a problem that could limit their therapeutic efficiency in the near future. Non-ethical practices to increase productivity and prevent diseases constitute another source of quinolone overuse (Adrian et al., 2009). Quinolone may enter into the food chain (Lara, 2006) since their residues often persist in edible tissues or foodstuffs. Adverse effects reported by long term exposure to quinolones include central nervous system (CNS) toxicity, phototoxicity, cardiotoxicity, arthropathy, and tendon toxicity (Chiffoleau et al., 2007; Dembry, Farrington, & Andriole, 1999; Hori, Yamakawa, Yoshida, Ohnishi, & Kawakami, 2012; Lara, 2006; Licata et al., 2012; Molloy & Mitry, 2012; Mulgaonkar, Venitz, & Sweet, 2012). For this reason, the employment of antibiotics such as quinolones in animal production is regulated and *maxim residue levels* (MRLs) have been established by the European Union (EC/470/2009, 2009; ECC/2377/90, 1990). Thus, the MRL for enrofloxacín (ERFX), including its metabolite ciprofloxacin (CPFX), is in the range of 100–300 μg kg^{-1} for different edible samples of animal origin.

Hair analysis is a useful alternative to improve the effectiveness of surveillance plans, as it offers several advantages compared to the analysis of other biological samples (liver, kidney, blood, etc.). Hair collection is a non-invasive procedure and offers a wider time window for drug detection due to its low metabolic activity. Therefore, drugs and their metabolites can be detected for a long time after treatment (Castellari, Gratacos-Cubarsi, & Garcia-Regueiro, 2009; Gratacos-Cubarsi, 2008; Henderson, 1993; Jeon, Kim, Paeng, Park, & Paeng, 2008). Since hair root is in contact with small blood vessels, drug residues can be incorporated in hair by passive diffusion from blood flow (Harkey, 1993). Incorporation from sweat, tallow or external contamination can also happen (Gaillard & Pepin, 1999; Nakahara, 1999). On the other hand, the zone of accumulation of residues varies because

* Corresponding author at: IQAC-CSIC, Jorge Girona, 18-26, 08034 Barcelona, Spain. Tel.: +34 93 4006100x5119; fax: +34 93 2045904.
E-mail address: pilar.marco@cid.csic.es (M.-Pilar Marco).
[1] This work is dedicated to the memory of Dr Francisco Sánchez-Baeza.

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of hair growth, so hair can act as permanent registration of drug residues (Henderson, 1993), which is an interesting property in order to know retrospective treatments.

The amount of antibiotics accumulated in hair is highly influenced by the type of hair, the dose administered or the melanin content. Thus, there has been observed differences in parameters such as the time of appearance, the permanence in the hair or the mechanism of drug incorporation (Dunnett, 2003; Durant et al., 2002; Gratacos-Cubarsi, 2008; Harkey, 1993; Henderson, 1993). As an example, Uematsu et al. (Uematsu, 1992, 1995), have investigated the time of appearance of ofloxacin in hair and the influence of the pigmentation. In the particular case of *quinolones*, ERFX and CPFX have been detected in the hair of horses (Dunnett, Richardson, & Lees, 2004), calfs and pigs (Gratacos-Cubarsi, 2007) by HPLC–FD (High Performance Liquid Chromatography with Fluorescence Detection). Quinolones are extracted from hair using different procedures. In most of them, a previous washing of the sample with solvents or surfactants is required and usually clean-up procedures have to be applied before HPLC analysis. As an example, a hydrolytic treatment with aqueous NaOH (80 °C, 30 min) followed by a purification step (solid phase extraction, SPE; or liquid–liquid extraction, LLE) has been reported for the extraction of ofloxacin, norfloxacin and CPFX from human hair (Mizuno, Uematsu, & Nakashima, 1994; Uematsu, 1991) and an acidic procedure with trifluoroacetic acid (TFA) followed by SPE purification has also been employed for ERFX and CPFX in animal hair (Dunnett et al., 2004).

Immunochemical analytical techniques have also been used to analyze distinct compounds in hair (see Table 1), particularly drugs of abuse in human hair, but their use to assess veterinary drug treatments on farm animals for human food consumption is very scarce. Few years ago, we demonstrated the potential of these technologies to assess treatment of farm animals with sulphonamide antibiotics (Adrian et al., 2009; Font et al., 2008), showing that residues of these antibiotics could readily be found and efficiently detected by ELISA in the hair of treated animals. On the other hand, some of us reported that ERFX residues could also be found in calf and pig hairs after the pharmacological treatment was started and that hair pigmentation enhanced quinolone accumulation significantly. Mean concentrations encountered by HPLC–FD for ERFX and CPFX ranged from 20 to 2518 ng g^{-1} in calves and from 152 to 1140 ng g^{-1} in pig (Gratacos-Cubarsi, 2007). With this precedents, in this paper, we report the implementation of a FQ broad-selectivity microplate-based ELISA (Pinacho, Sánchez-Baeza, & Marco, 2012), to the analysis of ERFX and CPFX in hair samples. To our knowledge, this is the first time that an immunochemical analytical procedure for the determination of fluoroquinolone antibiotic residues in hair of farm animals is reported.

2. Materials and methods

2.1. General methods and instruments

Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, DK). Washing steps were carried out on a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Shaking of the microtiter plates was done with Titramax 1000 (850 rpm) stirrer, Heidolph (Germany). Absorbances were read at a single wavelength mode of 450 nm on a spectrophotometer SpectramaxPlus (Molecular Devices, Sunnyvale, CA). The absorbance data was analyzed using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Sofware Inc., San Diego, CA). The pH and the conductivity of all buffers and solutions were measured with a pHmeter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Unless otherwise indicated, each standard concentration or sample was analyzed by ELISA in triplicate wells.

2.2. Chemicals, immunochemicals, buffers and solutions

Quinolones: EFRX and CPFX were supplied by Biochemika, Fluka (Italy). The 10 mM FQ stock solution was prepared in 50 mM $NaOH_{aq}$. The standard curves were prepared from this stock solution by serial dilutions in the assay buffer at concentrations ranging from 1000 to 0.025 nM). *Immunochemicals*: Preparation of the *coating antigen* (haptenized-BSA, FQ9-BSA) and production of the *polyclonal antisera* (As171, New Zealand white rabbit) have been reported (Marco, Sánchez-Baeza, & Pinacho, 2013; Pinacho et al., 2012). *Reagents and Buffers:* Phosphate-buffered saline, *PBS* is 0.01 M phosphate buffer, 0.8% saline solution, pH 7.5. *PBSx10* is PBS ten times more concentrated. *PBSCa* is PBS with 1 mM $CaCl_2$. *PBST2Ca* is 0.01 M PBS with 0.1% Tween 20, and 1 mM $CaCl_2$. *Coating buffer* is 0.05 M carbonate-bicarbonate buffer, pH 9.6. *PBSTCa* is 10 mM PBST, 1 mM $CaCl_2$, 0.5% Tween 20. *Citrate buffer* is 0.04 M of sodium citrate, pH 5.5. *Substrate solution* is 0.01% TMB (3,3′,5,5′-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. Reagents were supplied by Sigma Chemical Co. (St. Louis, MO), such as anti *IgG-HRP* (antirabbit goat IgG-HorseRadish Peroxidase).

2.3. Animal treatment and sampling

Hair Samples: Pigmented and unpigmented cattle hair samples were taken from animals reared at IRTA experimental farm (Institute for Food and Agricultural Research) and treated with quinolones, with (WP) and without (NoWP) withdrawal period, under the conditions described before (Gratacos-Cubarsi, 2007). Five pigmented (WP, $n = 2$; NoWP, $n = 3$) and six unpigmented (WP, $n = 2$; NoWP, $n = 4$) hair samples were obtained from two animals treated with an intramuscular ERFX daily dose (7.5 mg kg^{-1} live weight) during five days. The two control samples (one pigmented, sample 25; one unpigmented, sample 26) were obtained from one animal reared under the same conditions but not treated (*blanks)*. Samples 41, 42, 45 and 46 were taken after a withdrawal time of one week (WP2), samples 105, 108 and 111 after a withdrawal of four weeks (WP3), while samples 239, 241, 243 and 245 were obtained without withdrawal (NoWP2). The detailed description of ERFX administration, hair cutting, washing and sampling procedures has been previously described (Gratacos-Cubarsi, 2007). All samples were stored at −20 °C until analysis. Sample information is summarised in Table 2.

2.4. ELISA method

(1) *Coating step:* Microtiter plates were coated with the antigen FQ9-BSA solution (0.25 μg mL^{-1}, 100 μL/well) prepared in coating buffer at room temperature (RT) for 4.5 h and covered with adhesive plate sealers. The plates were washed automatically four times with PBST. *(*2) *Competence step:* Solutions of the ERFX standards (1000–0.025 nM, and zero concentration in PBSCa) or the samples were added to the microplates (50 μL/well), followed by the As171 solution (1/64000, in PBST2Ca buffer, 50 μL/well) and incubated for 30 min at RT. Plates were washed as before. (3) *anti-IgG-HRP addition:* a solution of anti-IgG (1/6000 dilution in PBST, 100 μL/well) was added to the plates and after 30 min incubation at RT, the plates were washed again. (4) *Substrate reaction:* the substrate solution was added (100 μL/well) and left for 30 min at RT. Coloured product formation was stopped with 4 N H_2SO_4 (50 μL/well). All the steps were developed under shaking (900 rpm) except for coating step. *Signal processing: Ab*sorbance of each well was read at 450 nm. Unless otherwise indicated, data correspond