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COMUNICACIONES

Simultaneous quantification of ciprofloxacin, enrofloxacin and balofloxacin in broiler chicken muscle

Cuantificación simultánea de ciprofloxacina, enrofloxacina y balofloxacina en músculo de pollo parrillero

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RESUMEN

La presencia de residuos de medicamentos veterinarios en alimentos es una preocupación creciente para consumidores y para entes reguladores internacionales. Los límites máximos de residuos (LMR) en tejidos comestibles de origen animal son establecidos a partir del desarrollo de métodos analíticos. Se efectuó la validación del método de detección de ciprofloxacina (CFX), enrofloxacina (EFX) y balofloxacina (BFX) en músculo de pollos parrilleros por extracción en fase líquida y determinación simultánea por cromatografía líquida de alta presión (HPLC). Las muestras se homogenizaron y se adicionaron inicialmente con los antimicrobianos, luego se extrajeron con diclorometano dos veces. Estas fluoroquinolonas fueron cuantificadas por HPLC, detección por fluorescencia a 295 nm de excitación y 500 nm de emisión. El ensayo fue linear entre 0.001 y 0.1 µg/g.

Los límites de cuantificación fueron 0.0119 µg/g para EFX, 0.0436 µg/g BFX y 0.0557 µg/g para CFX. Los índices de recuperabilidad de los metabolitos en músculo, entre 0.01 y 0.1 ug/g, calculados comparando las muestras inicialmente adicionadas contra muestras de músculo con incorporación de los antimicrobianos luego del procesado, promediaron los 77.47% para EFX, 87.7% para CFX y 96.67% para BFX. Los extractos eluidos no presentaron interferencias de la matriz biológica y tuvieron buena resolución de los analitos en los cromatogramas. Los LMR son establecidos a partir de los factores de riesgo, ensayos toxicológicos pertinentes y métodos de detección desarrollados y validados, los cuales deben ser factibles de implementación. Este es un método simple y económico para cuantificar la presencia simultánea de CFX, EFX y BFX en músculo de pollo.

Palabras claves: enrofloxacina, ciprofloxacina, balofloxacina, músculo, HPLC.

Key words: enrofloxacin, ciprofloxacin, balofloxacin, muscle, HPLC.

INTRODUCTION

Ciprofloxacin (CFX) and enrofloxacin (EFX) are second generation fluoroquinolones with a broad spectrum of antibacterial activity. Both have good bioavailability after oral administration and good to excellent tissue distribution (Papich, 1998). The interest of the medical community in fluoroquinolones has not decreased during the last 10 years and many new ones have been developed and are under investigation. Balofloxacin (BFX) 1-cyclopropyl-6-6fluoro-1, 4-dihydro-8-methoxy-7- (3-methylaminopiperidin-1-yl)-4-oxoquinoline-3-carboxylic dehydrated acid is a new, not commercialized one. Pharmacokinetic, microbiological, clinical and residue studies are needed for the pharmaco- toxicological evaluation of a new and alternative antimicrobial drug.

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EFX is intensively used in our country in poultry and pig production for preventive and therapeutic purposes in Argentina. CFX has been used for the first time into human medicine, being effective in several infections. Because of its broad and intense activity against Gram negative bacteria and the fact that no cross- resistance with beta- lactams or aminoglycosides occurs, it was also suggested for veterinary medicine use (Nouws et al., 1988). It is proposed to be used in dogs and cats (Brown, 1996) but not in food producing animals. Due to the antibacterial advantages and pharmacokinetic properties reported, its clinical application in veterinary medicine could be of considerable usefulness. There are reports of pharmacokinetics of CFX in domestic animals (Aramayona et al., 1996; Dowling et al., 1995; García Ovando et al., 2000a; García Ovando et al., 1999) showing good pharmacokinetic properties and therapeutic possibilites.

Following the introduction of fluoroquinolones for use in poultry, there has been a dramatic emergence of Salmonella with reduced susceptibility to fluoroquinolones in humans (WHO, 1997; WHO, 1998). This fact marks the importance to develop new methods for a fast, simple and accurate quantification of residues of these antibacterials in food producing animals. Maximum Residue Limits (MRL) in animal products and sub-products of human consume are required by control regulations for the protection of the consumers.

The objective of this work was to quantify simultaneously CFX, EFX and BFX in chicken muscle.

MATERIAL AND METHODS

*Apparatus***.** The apparatus used for the High Pressure Liquid Chromatography (HPLC) analysis was a Hewlett Packard (HP) 1050 multisolvent delivery system, equipped with a HP 1050 fluorescence detector. Peak ratios were recorded with a HP Integrator. Separation was carried out at room temperature on a reverse-phase Phenomenex aqua 5µ C_{18} column (150 x 4.60 mm, 5 µm particle size). A Phenomenex security guard column C18 ODS, octadecyl, 4 mm L x 30 mm ID, was used to ensure the lifetime of the column.

Reagents and Materials. (a) Antimicrobial standards: Ciprofloxacin from Parafarm, Argentina; Enrofloxacin from Laboratorios Recalcine, Chile; Balofloxacin is a gift from Dr Nakagawa, Chugai Pharmaceutical Co. Ltd., Japan. (b) Water-distilled, de-ionized and filtered through 0.45 µm. (b) Buffer phosphate solution 0.1M, pH 7.2. (c) Stock standards: 500 µg/ mL EFX, CFX and BFX solutions were prepared monthly in deionized and bidistilled water, EFX and BFX with drops of ClH 0.1M until complete dissolution; all were stored at 4^0 C. (d) Working standard solutions: Dilutions of stock standards to obtain 0.00375, 0.0075, 0.015, 0.03, 0.06, and 0.1 µg CFX, EFX, or BFX/ g of muscle were prepared daily. (e) Muscle samples: Chickens were purchased from a local farmer and were analyzed for fluoroquinolones absence before work. (f) Solvents: Dichloromethane, acetonitrile and triethylamine HPLC grade (Sintorgan).

Chromatography conditions. The mobile phase used was water. acetonitrile. triethylamine (80.19.1), adjusted to pH 3.0 with phosphoric acid. The mobile phase was filtered through 0.45 µ nylon membrane before use. The flow rate employed was 1.2 mL/ min with a fluorescence detection at 295 nm excitation wavelength and 500 nm emission wavelength, lamp 3, PMT gain 9, response time 6. These conditions permitted the correct detection of the three antimicrobials, ciprofloxacin, enrofloxacin and balofloxacin.

Extraction procedure. The muscle (0.2 g) was homogenized with phosphate buffer (2 mL). Dicholoromethane (8 mL) was added to the homogenate, vortexed for 1 min and centrifuged at 4.000 rpm for 20 min. The upper aqueous layer was discarded, the organic phase was transferred to a clean tube and the tissue was again extracted with 6 mL of dichloromethane. Organic layers were combined and evaporated at 30 ^{0}C under nitrogen stream. The extract was redissolved with 200 µL of mobile phase and 100 µL was used

foo HPLC analysis. The quantification in µg of antimicrobial/ g of muscle was performed in relation to the correspondent peak area of the standard curve.

Standard curve and Linearity. Homogenized muscle was added with the antimicrobials to render a standard curve: 0.01, 0.03, 0.06 and 0.1 µg/g. The samples were then treated according to the described extraction procedure. Results expressed as peak area of CFX, EFX and BFX versus the correspondent concentrations were analyzed for linearity. The limit of quantification was considered the concentration of antimicrobials detected with a signal to noise ratio of 10 (10x standard deviation of blanks/ standard curve slope). The limit of detection was considered the concentration of antimicrobials detected with a signal to noise ratio of three.

Recovery. Working standard CFX, EFX and BFX solutions were added to the re-dissolved extracts of blank muscle samples processed with the described extraction procedure to obtain 0.03, 0.01 and 0.1 µg/ mL. This was considered the 100% of antimicrobial concentration in the muscle. The recovery of antimicrobial residues from the muscle was calculated through the diminution of the antimicrobial peak area in the initially added muscle, in relation to the 100% concentrations. It is expressed as % of recovery.

RESULTS AND DISCUSSION

Chromatography. Residues were detected by HPLC using fluorescence detection and a typical chromatogram of chicken muscle with the three antimicrobials is shown in Figure 1.

CFX elutes at 2.658 ± 0.28 min, EFX at 3.374 ± 0.15 min and BFX at 7.239 ± 0.39 min.

Linearity. Linear correlation graphs were obtained in the range of 0.01 to 0.1 µg CFX-EFX- BFX/ g of chicken muscle. Correlation coefficient was 0.955 for CFX, 0.976 for EFX and 0.965 for BFX.

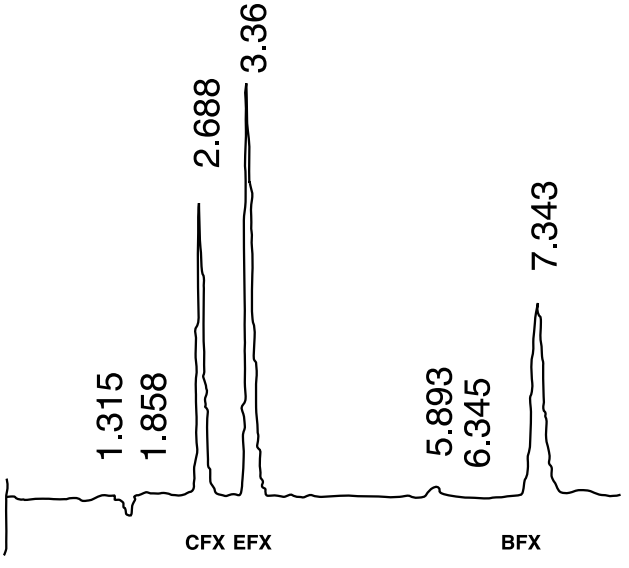

FIGURE 1. Typical chromatogram of chicken muscle with 0.03 µg of ciprofloxacin (retention time rt: 2.688 min), enrofloxacin (rt: 3.365 min) and balofloxacin (rt: 7.345 min)/ g of tissue.

Cromatograma típico de músculo de pollo con 0,03µg de ciprofloxacina (tiempo de retención rt: 2.688 min), enrofloxacina (rt: 3.365 min) y balofloxacina (rt: 7345 min)/ g de tejido.

Limit of quantification and limit of detection. The limit of quantification in chicken muscle was 0.0119 µg/g for EFX, 0.0436 µg/g for BFX and 0.0557 µg/g for CFX. The limits of detection in chicken muscle was 0.0036 µg/g for EFX, 0.0109 µg/g for BFX and 0.0182 µg/g for CFX.

Recovery. The recovery of the antimicrobials from the muscle at 0.01- 0.03 and 0.1 µg/g were 103.5, 59.9 and 99.9 % for EFX, 101.6, 58.3 and 72.5% for CFX, and 122.1, 91.5 and 76.4% for BFX.

In May 1997, the Food and Drug Administration published an order of prohibition against the extra label use of fluoroquinolones in food- producing animals. It is believed that some uses are capable of increasing the level of drug resistant zoonotic pathogens in treated animals at the time of slaughter (FDA, 1997). In Argentina EFX is specially used in chickens and pigs, giving the importance of a rapid detection of fluoroquinolones in these meats. It is also important to detect CFX, the product of EFX metabolism. In studies performed in different animal species, CFX was detected in concentrations from 10%- 35% to concentrations equivalent to, or higher than, those of the parent drug (Anadón *et al.,* 1995; Mengozzi *et al.,* 1996; Intorre *et al.,* 1997; Nielsen *et al.,* 1997; García Ovando *et al.,* 2000a). Moreover, CFX is a good present or future alternative for use in food producing animals as it has shown quick elimination from chicken bodies with minor possibilities of leaving residues in treated animals (García Ovando *et al.,* 1999). In a previous work, we have also analyzed the presence of residues of EFX and CFX in eggs of laying hens, and a minor withdrawal time was determined for CFX than for EFX at an identical dosage regime (Gorla *et al.,* 1997). On the other hand, pharmacokinetic characteristics of BFX were good absorption and distribution in rats and dogs, similar to other fluoroquinolones, which should be advantageous for the treatment of various animal infections (Nakagawa *et al.,* 1995).

Liquid- liquid extraction has been useful for the determination of enrofloxacin in eggs (Gorla *et al.,* 1997), bovine milk (Tyczkowska *et al.,* 1994), and chicken muscle (García Ovando *et al.,* 2000b, Yorke and Froc, 2000). Yorke and Froc have quantified nine quinolones in chicken muscle using three different HPLC conditions. We have now performed the analytical validation for the simultaneous quantification of CFX, EFX and BFX, with a good resolution of the three antimicrobials. The present method lasted three hours from the reception of the tissue to the analysis of the sample, with good recovery, linearity, and limit of quantification and it is feasible for laboratories, avoiding the importation of cartridges of solid phase extraction.

The mean % of recovery obtained for CFX (77.47%), EFX (87.77%), and BFX (96.67%) are in the order of the recommended recoveries of the analytes by the Member States of the Commission of the European Communities, 45-100% (Heitzman, 1994). The Food and Drug Administration, established a tolerance of 0.3µg/g for residues of EFX in muscle of chickens and turkeys (FDA, 2002)[1]. The MRL permitted by the European agency for the evaluation of medicinal products, Committee for veterinary medicinal products, for enrofloxacin and its metabolite ciprofloxacin are 100- 300 ug/ Kg in muscle, liver and kidney of bovine, porcine, rabbit, ovine and poultry species, and 100 ug/ Kg for bovine milk (EMEA, 1998a). In the same way the Codex Alimentarius Comission, an organ created by the FAO and the WHO for developing security standards in food, does not determine MRL for fluoroquinolones, being danofloxacin and sarafloxacin under evaluation with tentative tolerances of 0.2 ug/g and 0.01 ug/g respectively (Codex, 1999). The limits of quantification of the present assay are below those MRL. The present method, of excellent feasibility for developing countries, has permitted the simultaneous

1 FDA, Food and Drug Administration Department of Health and Human Services 2002. Approved Animal Products On Line Database System http://dil.vetmed.vt.edu/cfr/CFR.cfm.
EMEA The European Agency for the Evaluation of Medicinal products, Committee for veterinary medicinal products. Enrofloxacin (modification for bovine, porcine and poultry), Summary report (2), EMEA/MRL/388/98, 1-6. http://www.eudra.org/emea.html, 1998.

detection of ciprofloxacin, enrofloxacin and balofloxacin with good resolution and specificity, and acceptable sensitivity. Consumers and authorities are concerned about the control of veterinary drug residues in farm animals and it is necessary to have accurate and simple methods to quantify them.

SUMMARY

There is an increasing public concern about the presence of veterinary residues in food. The Maximal Residue Limit (MRL) in edible tissues of animal origin are established from the analytical methods developed. The extraction and simultaneous liquid chromatographic quantification of ciprofloxacin (CFX), enrofloxacin (EFX) and balofloxacin (BFX) was validated in muscle of broiler chicken. The samples were homogenized and the antimicrobials added, then they were extracted twice with dichloromethane. These fluoroquinolones were detected by liquid chromatography with fluorescence at 295 nm excitation and 500 nm emission. The assay was linear from 0.001 to 0.1µg/ mL. The limits of quantification in chicken muscle were 0.0119 µg/g for EFX, 0.0436 for BFX and 0.0557 for CFX. The indices for the recoveries of residues (0.01 to 0.1 ug/ mL) were calculated in comparison to samples with the incorporation of these antimicrobials after the extraction procedure, averaged 77.47% for EFX, 87.7% for CFX and 96.67% for BFX. The extracts were free of interference from the biological matrix, with good resolution of the peaks in the chromatograms. The MRL are usually established from the risk factors, pertinent toxicological assays and developed analytical methods, which have to be able for implementation. This is a simple and economic method to quantify the presence of CFX, EFX and BFX in chicken muscle.

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