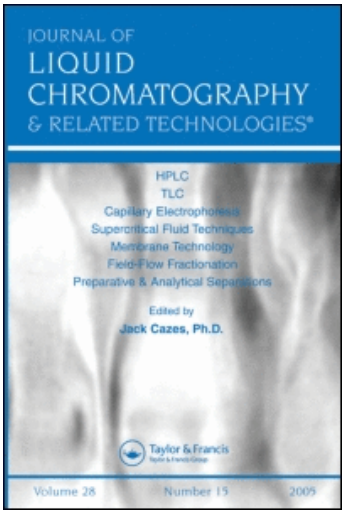

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ENROFLOXACIN LIQUID-LIQUID EXTRACTION FROM CHICKEN MUSCLE AND HPLC DETECTION

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ABSTRACT

A technique for the extraction and liquid chromatographic determination of enrofloxacin residues in chicken muscle is described. The muscle samples were extracted with three steps of dichloromethane and centrifugation for the isolation of enrofloxacin from muscle.

Enrofloxacin was quantified by liquid chromatography with fluorescence detection at 297 nm excitation and 440 nm emission. The assay is linear from 0.00048 to 0.25 μg/mL. The limit of detection is 0.00048 μg/mL and the limit of quantification is 0.0039 μg/mL. The recoveries of enrofloxacin residues (0.0039 to 0.25 μg/mL) averaged 86.67%. The injected extracts are free from matrix interferences, making it easy to calculate the amount of residue present.

This is a sensitive, economic, and fast method to investigate the presence of enrofloxacin in chicken muscle.

INTRODUCTION

The enrofloxacin (EFX) is a fluoroquinolone, derived from the original quinolone, nalidixic acid. Many of these antimicrobials are zwitterions and exhibit different solubility characteristics with changes of pH. These agents are generally quite stable in both oral and parenteral dosage forms at or below 30°C. They have also appeared to be stable in reconstituted serum and urine for several weeks. The compounds are sensitive to strong light and should be protected from light for long-term storage to prevent loss of activity.[1]

EFX has many advantages, such as, a spectrum of activity that includes most Gram-negative bacteria and many Gram-positive bacteria, including staphylococci It has excellent tissue penetration, and it is relatively safe.[2]

The sector of animals' production uses EFX for preventive and therapeutic purposes. Experts of the World Health Organization have reported that, following the introduction of fluoroquinolones for use in poultry, there has been a dramatic rise in the prevalence of fluoroquinolone-resistant Campylobacter in poultry, and infections in humans in many countries, and the emergence of Salmonella with reduced susceptibility to fluoroquinolones in humans.[3,4]

For the protection of the consumers it is important to develop methods for easy quantification of antimicrobials in foods of animal origin, and with this objective, maximum residue limits (MRL) have been established for the chemotherapeutics, according to European Union (EU) and United States of America regulations.

EFX is extensively used in our country in chickens and pigs. The objective of this work was to develop a simple and economical method to determine EFX residues in chicken muscle.

EXPERIMENTAL

Apparatus

The apparatus used for the HPLC analysis was a Hewlett Packard (HP) 1050 multisolvent delivery system, equipped with a HP 1050 fluorescence detector. Peak ratios were recorded with an HP integrator. Separation was carried out at room temperature on a reverse-phase HP ODS C_{18} column (4.6 x 200 mm, 5 μm particle size). A guard column (cartridge holder and guard cartridge ODS Hypersil 20 x 4 mm, 5 μm) was used to reduce contamination of the analytical column.

Reagents and Materials

Solvents: Dichloromethane, acetonitrile, and triethylamine HPLC grade (Sintorgan). Water: Distilled, deionized, and filtered. Buffer: phosphate solution 0.1M, pH 7.2. Analytical standard: enrofloxacin from Laboratorios Recalcine, Chile. Stock standard of 500 μg/mL EFX solution was prepared monthly, in deionized and bidistilled water, with drops of HCl 0.1M until complete dissolution and stored at 4°C. Working standard EFX solutions: Dilutions of stock standard EFX for a 7-points standard curve to obtain 0.0048, 0.0077, 0.015, 0.031, 0.063, 0.125, and 0.250 μg enrofloxacin/g of muscle were prepared daily. Muscle samples: Muscle samples were obtained from chickens of the University farm, fed with commercial chickenfeed, free from fluoroquinolones.

Extraction Procedure

In a 20 mL homogenizer, 1 g of muscle was homogenized with 4 mL of phosphate buffer. Eight mL of dichloromethane was added to the homogenate and shaken for 2 min and then centrifuged at 10,000 rpm for 10 min. The solvent layer was transferred to a fresh tube and re- extracted with 3 mL of dichloromethane by shaking another 2 min, and further centrifuged. Solvent layers were combined and filtrated through a phase separator silicone-treated filter (Whatman) to clean the fraction, and then evaporated at 50°C in a thermostatic bath, into a fume gas cabinet. A 500 μL aliquot of mobile phase was added to the tubes to re-suspend the extract. It was filtrated through a 0.45 μ nylon membrane, and 100 μL of filtrate was injected into the column for HPLC analysis. Quantification of EFX in μg/g of muscle was based on peak area measurements.

Calibration Curve

Aliquots of 25 μL of the working standards EFX solutions were added to 1 g of buffered homogenized muscle. These samples were then treated according to the described extraction procedure. Results expressed as peak area of EFX versus concentrations of EFX were tested for linearity with a coefficient of correlation. The limit of quantification was considered the lowest concentration of EFX detected with a signal to noise ratio of 3. The limit of detection was the lowest concentration of EFX detected with a signal to noise ratio below 3.

Curve of Tissue

Working standard EFX solutions of 0.0039- 0.0310 and 0.250 μg/g were added to the re-suspended extracts of blank muscle samples and processed with the described extraction procedure, to construct the curve of added tissue.

Recovery

The recovery of EFX residues from the muscle was calculated through the diminution of EFX area in the calibration curve, considering as 100% the area obtained in the curve of the added tissue. It is expressed as % of recovery.

Chromatography

The mobile phase was a water- acetonitrile- triethylamine (80:19:1). The pH was adjusted to 3.0 with phosphoric acid. This mixture was filtered through 0.45 μ nylon membrane prior to use. A flow rate of 1.5 mL/min was employed with fluorescence detection at 297 nm excitation wavelength and 440 nm emission wavelength, response time 6, lamp 3, and PMT gain 15, which provided optimum absorbance for maximal detection.

RESULTS

Chromatography

Residues were detected by liquid chromatography with fluorescence detection, and representative chromatograms of control muscle samples showed no interfering matrix background peaks (Figure 1). EFX elutes at 4.0 ± 0.15 min.

Validation of the Extraction Method

Linearity and Quantitation Limit

Linear correlation graphs were obtained in the range of 0.00048 to 0.25 μg enrofloxacin/g of chicken muscle. The correlation coefficient was 0.998. The limit of quantification of EFX in muscle was 0.0039 μg/g and the limit of detection was 0.00048 μg/g.

Recovery

The recoveries of enrofloxacin residues from the muscle (0.0039, 0.0310 and 0.250 μg/g) were 71%, 89%, and 100 %, respectively.

Precision

The day to day precision of the proposed method was studied by performing the recoveries and EFX peak area versus concentration on three different

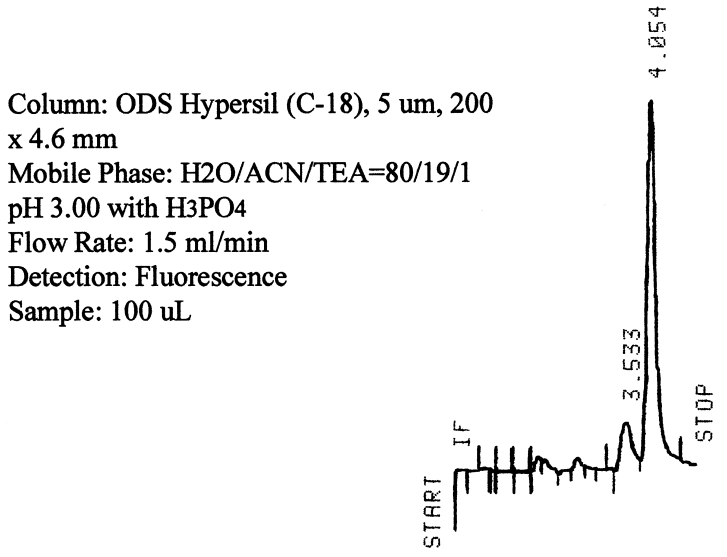

Figure 1. Enrofloxacin typical chromatogram in chicken muscle; retention time: 4.054 min.

days. The coefficients of variation were 8.07% for an averaged % of recovery and 3 % for the areas.

DISCUSSION

A method for the liquid-liquid extraction and fluorescence chromatographic determination of enrofloxacin residues in chicken muscle was validated.

Many reports in the literature employ solid phase extraction (SPE) clean up and HPLC detection for the determination of residual enrofloxacin in meat and fish,[5] bovine and porcine muscle,[6] and SPE plus thin layer chromatography in pig muscle.[7] In a parallel manner, liquid- liquid extraction has been useful for the determination of enrofloxacin in eggs,[8] chicken muscle,[9] bovine milk,[10] and egg and poultry meat.[11]

Initially, we explored SPE clean up using commercially available cartridges. The efficiency of the SPE column cleanup was evaluated in comparison with liquid-liquid extraction, in terms of interferences, variability, and especially, feasibility for our country. The liquid-liquid extraction was far supe-

rior with minor variability and economic advantages, so we have performed the analytical validation of this extraction method. The present method requires 3 h from the reception of the tissue to obtaining of the results, with good recovery, linearity, limit of quantification, and precision.

Recovery of the analyte should fall within the range of 45- 100%.[12] Our average 86.67% of recovery of enrofloxacin, is in the range of 51.3- 74.8% obtained for pig muscle and 74.9- 90.2% reported for bovine muscle with SPE extraction.[12]

Consumers and authorities are concerned about the examination of animals and fresh meat for the presence of residues, an important issue in public health. There is an increasing vigilance and control of residues of veterinary drugs in farm animals, and it is necessary to have adequate technical resources to carry out the legislation.

Maximum residue limits (MRL) are established from the limits of quantification of the techniques used. Improving methods of residue detection to be used in routine analysis, will contribute to make laws relevant for residues control.

The routine screening method for the determination of ciprofloxacin and enrofloxacin in pig and bovine muscle, proposed by Member States of the Commission of the European communities, has a limit of detection of 0.01 μg/g of tissue.[12] The MRL for enrofloxacin in the EU is 30 ng/g for kidney, liver, and muscle, in swine, cattle, and poultry.[13] The detection limits of quantification of the present assay, 3.9 ng/g, is below the MRL stipulated by the EU.

New analytical technology is in a state of a continuous change and progress, but this traditional method with multiple solvent extraction and centrifugation steps for the isolation of EFX from muscle, is of acceptable quality and ideally adapted to the reality of our country, without the necessity of imported cartridges. It is fast, economical, and sensitive.

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