JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of the Fluoroquinolone Enrofloxacin in Edible Chicken Muscle by Supercritical Fluid Extraction and Liquid Chromatography with Fluorescence Detection

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A supercritical fluid extraction (SFE) method for the extraction of enrofloxacin from a chicken breast muscle was examined. A liquid chromatograph, equipped with a fluorescence detector, was used for the detection of enrofloxacin. Optimal extraction parameters, such as extraction time, supercritical fluid volume, modifier concentration, pressure, and temperature, were determined by examining SFE recoveries from control muscle samples spiked with enrofloxacin at different levels. In all of the experiments, high recovery values were observed, ranging from 101 to 104%. The extraction of enrofloxacin from real muscle samples was examined in chickens that were treated orally with enrofloxacin. Extraction was carried out by the SFE method after each oral treatment and under optimal extraction conditions at set intervals over time. The SFE, combined with liquid chromatographic analysis, showed that the concentration of enrofloxacin in the chicken muscles decreased continuously with time, giving a negligible concentration 72 h after the treatment. These results suggest that SFE is a useful approach for the extraction of enrofloxacin from chicken breast muscles.

KEYWORDS: Enrofloxacin; fluoroquinolones; supercritical fluid extraction

INTRODUCTION

Enrofloxacin is one of the third-generation members of the fluoroquinolone antibacterial agents that are effective in controlling a wide range of bacteria in animals. It is effective against microorganisms that are resistant to other antibacterial agents such as aminoglycosides, tetracyclines, macrolides, and β-lactam (*1*). The antibacterial effectiveness of enrofloxacin contributes to the widespread use of this agent in food animals. Enrofloxacin is known to rapidly penetrate body tissues and fluid (*2*, *3*) and has been detected in animals (*4*−*7*). The presence of antibacterial agents in food animals could induce pathogen resistance in humans (*8*−*10*). Thus, a growing concern over the presence of enrofloxacin in animals has triggered scientific activities into a closer examination of the detection methods used to monitor this agent.

Liquid chromatographic methodologies are known to be among the most sensitive methods to detect enrofloxacin in biological samples (*11*−*15*). Liquid chromatography−mass spectrometry techniques have been well documented as sensitive methods for the multiresidue analysis of fluoroquinolones in chicken and eggs (*16*, *17*). Most of these methods are generally based on solvent extraction methods, in which samples are usually blended into organic or aqueous−organic solvents. In the solvent extraction methods, the extraction of enrofloxacin from biological samples is a major challenge because this agent has two ionizable functional groups, such as acidic carboxyl and basic piperazinyl. These chemical groups are involved in pH-dependent interactions between fluoroquinolones and biological sample matrices (*18*−*20*). This can result in poor extraction, isolation, and purification of antibacterial agents from the biological samples, which is well documented in research involving biological lipoprotein samples, such as eggs. Eggs are a difficult biological sample to analyze because their lipoprotein matrices have the potential to form emulsions and foams when they come into contact with the extraction solvent (*20*, *21*). Solvent extraction methods, therefore, require solid phase extractions, such as ion exchange, adsorption, or reversed phases, for the isolation of antibacterial agents from biological samples.

To avoid the limitations of solvent extraction, supercritical fluid extraction (SFE) has been introduced as an alternative method. SFE is more economical and environmentally safer than conventional extraction, using a solvent. SFE allows researchers

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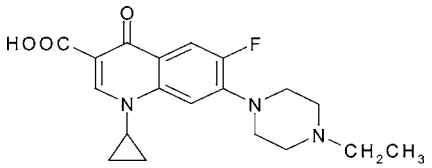
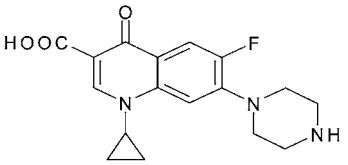
10.1021/jf0346511 CCC: $25.00
Published on Web 11/21/2003



Enrofloxacin



Ciprofloxacin

Figure 1. Chemical structures of the fluoroquinolones tested.

to skip the experimental steps, such as solvent extraction and chromatographic cleanup, that are usually required after solvent extraction. Considering the experimental problems that may arise during extraction, SFE is suggested as a promising method for the extraction of fluoroquinolones from biological samples. Our laboratory recently reported a suitable method for the determination of fluoroquinolones in eggs, using a combination of SFE and liquid chromatographic analysis (*22*). In the previous study, fluoroquinolones could be extracted from egg samples using SFE, giving high recovery values close to those of a solvent extraction method. Lipoprotein matrices that could result in the poor extraction of fluoroquinolones were initially removed using SFE with supercritical CO_2. Fluoroquinolones were then successfully extracted using supercritical CO_2 with a small amount of methanol as a modifier. When the SFE method was used, no interference in the liquid chromatographic analysis was observed in the control samples, suggesting that SFE was efficient in the extraction of fluoroquinolones from eggs.

In the present study, we examined a SFE method for the extraction of enrofloxacin from chicken breast muscles. Chickens are a favorite food animal during the summer season in Korea. Thus, monitoring the residue of enrofloxacin in chickens is of great interest in food safety. The present paper is mainly devoted to the application of the SFE method for the extraction of enrofloxacin from chicken breast muscles. Chickens were orally treated with enrofloxacin, and their muscles were extracted using SFE at set intervals so as to determine the level of enrofloxacin over time. This is the first known method of SFE of enrofloxacin from orally treated chickens.

MATERIALS AND METHODS

Chemicals. Enrofloxacin (99.2%) was purchased from Merck (Darmstadt, Germany). Ciprofloxacin (100%) was purchased from Jailjedang (Seoul, Korea). All solvents used in this study were obtained from Yakuri (Japan). All chemicals and solvents used in this study were of analytical grade, unless otherwise stated. The chemical structures of enrofloxacin and ciprofloxacin are presented in **Figure 1**.

Apparatus. SFE was performed by using a Jasco model PU980 dual pump (Tokyo, Japan) with one pump for pure CO_2 and the other for methanol. The pumps were connected to a 10-mL stainless SFE vessel (10 mm o.d. × 15 cm length) in a column oven chamber that was directly connected to a Jasco model 880-81 pressure restrictor. The flow rate and pressure of the supercritical fluid was maintained using a pressure restrictor. A Shimazdu model LC10AD high-performance liquid chromatograph (HPLC) equipped with a Shimadzu model RF LC10A fluorescence detector was used. Fluoroquinolones were detected at a 278-nm excitation wavelength and a 450-nm emission wavelength. Sample analytes were separated on a Waters NovaPak C_{18} stainless steel column (particle size = 4 μm, 3.9 i.d. × 300 mm) by a mobile phase, consisting of 4 mM phosphoric acid/methanol/triethylamine (70:25:5, v/v/v, pH 3.5), with a flow rate of 1.0 mL/min, as described previously (*22*).

Optimization of Extraction Conditions. The extraction parameters, such as extraction time, supercritical fluid volume, modifier concentration, pressure, and temperature, were optimized by examining the recovery of enrofloxacin from spiked freeze-dried muscle samples. The muscle samples were obtained from antibiotic-free chickens. Two-gram (wet weight) portions of the samples were transferred to a series of freeze-dryer vessels and dried using a model SFDSM12 Samwon freeze-dryer (Busan, Korea). The moisture content of the samples was determined by measuring the weight differences before and after freeze-drying. Enrofloxacin prepared in methanol was added to the freeze-dried samples at a concentration of 200 ppb. Only a solvent was added to the control sample. The spiked samples were transferred to the SFE vessel after the solvent had evaporated. The samples were then extracted using the SFE, with only supercritical CO_2 or supercritical CO_2 containing methanol, as a modifier, and the concentration of methanol was increased with each sample. Extracts were collected every 10 min for up to 50 min of total extraction time by inserting the outlet of the SFE restrictor into a round-bottom flask, preheated to 40 °C and containing 10 mL of methanol. The solvent was evaporated at 40 °C using a vacuum evaporator, and the residue was dissolved in 10 mL of a solvent mixture of phosphoric acid/methanol (1:1, v/v) mixture. The mixture was then centrifuged at 3000 rpm at 4 °C for 10 min, using a benchtop centrifuge. The supernatant was then used for HPLC analysis. Eenrofloxacin concentrations in the samples were calculated from the standard calibration curve. The experiments were carried out in triplicate.

A recovery test was also performed by examining the extraction of enrofloxacin from fortified muscles. Control muscles were obtained from antibiotic-free chickens as described above. Two-gram (wet weight) portions of triplicate muscle samples were placed on aluminum foil. Enrofloxacin prepared in methanol was added uniformly to the samples, at concentrations of 50 and 100 ppb before freeze-drying. The samples were allowed to stand in a ventilated hood for solvent evaporation. The samples were then transferred to the freeze-dryer vessels and freeze-dried as previously described. The freeze-dried samples were then transferred to the SFE vessels and extracted using the SFE, at the optimal extraction conditions determined above. The extracts were collected and prepared for HPLC analysis as described above.

Quantitation and Detection Limits. The concentrations of enrofloxacin in the samples were calculated on the basis of wet sample weight throughout the experiments. All of the samples were injected into the HPLC in 20-μL volumes. Enrofloxacin was determined by comparing its retention time to that of a standard working solution on the HPLC column. The stock solution of the standard enrofloxacin was prepared by dissolving 10 mg of standard in 100 mL of methanol, to reach a final concentration of 100 ppm. The working solutions for the HPLC injections were prepared from serially diluted stock solution in a solvent mixture of phosphoric acid/methanol (1:1, v/v). The standard calibration curve was obtained by injecting five-level concentrations ranging from 2.5 to 50 ppb and measuring the peak areas of the chromatograms. The limit of detection (LOD) of the smallest amount of enrofloxacin was calculated on the basis of a signal-to-noise (S/N) ratio of 3:1. The S/N ratio was obtained by referring to the peak height of the 1 ppb enrofloxacin standard. The limit of quantitation (LOQ) was calculated as follows: $A \times 1/B \times D/C$, where A represents the LOD (ng), B represents the HPLC injection volume (μL), and C and D represent the sample amount (g, wet wt basis) and final dilution volume (mL), respectively.

Animal Treatment. We attempted to determine enrofloxacin in chicken breast muscles of chickens orally treated with enrofloxacin. Four-week-old chickens with no history of fluoroquinolone treatment were used. The chickens were subjected to experimental conditions for at least 1 week. The chickens were starved for 12 h prior to enrofloxacin treatment, and their body weights were measured to calculate the treatment dose of enrofloxacin. Enprotil (Eaglevet, Seoul, Korea), a commercial product of enrofloxacin available in Korea, was

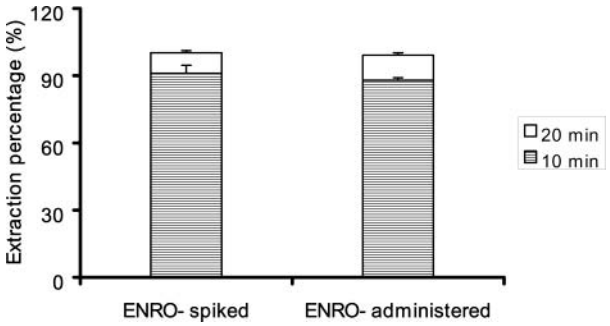


Figure 2. Time course percentage values for the extraction of enrofloxacin using SFE from the spiked freeze-dried chicken muscles (ENRO-spiked) and the orally treated chicken breast muscles (ENRO-administered) with enrofloxacin. The data given are the means ± SD of triplicate measurements.

prepared in the drinking water at a dose of 10 mg of active ingredient/kg of body weight, which was the dose level recommended by the manufacturer. Feed was provided normally without enrofloxacin. The drinking water was supplied in a water vessel at a ratio of 60 mL a day. The chickens had free access to both feed and water. The treated drinking water was replaced by enrofloxacin-free water 6 h after the treatment, and three chickens were removed at set intervals for samples. Breast muscle samples were obtained from the chickens and extracted using SFE for the time course determination of enrofloxacin. Samples were diluted with the solvent mixture in case enrofloxacin in the samples was found to be off the standard calibration curve, and concentrations were back-calculated by multiplying a dilution factor. All experiments were carried out in triplicate, unless otherwise stated.

RESULTS AND DISCUSSION

We recently reported a SFE method for the extraction of fluoroquinolones from a biological sample (*22*). Our main contribution in the present study was to examine the application of SFE for the extraction of enrofloxacin from edible chicken muscles.

Figure 2 shows the extraction of enrofloxacin from the freeze-dried muscles spiked with enrofloxacin at 200 ppb. The optimal extraction condition was using 20% (v/v) methanol for the modifier concentration and 60 °C and 300 atm for the SFE apparatus. As a result, these conditions were used for sample extractions throughout this study. When enrofloxacin was extracted using supercritical CO_2, containing 20% (v/v) methanol, ~91% of the enrofloxacin originally spiked in the freeze-dried samples was extracted within the first 10 min. Nearly complete extraction was achieved after a further 10 min, giving 20 min of total extraction time. To show that the same extraction results could also be obtained from orally dosed chicken muscles, the muscle samples were extracted 24 h after the treatment. More than 87% of the total enrofloxacin found in the muscle samples could be extracted using SFE within the first 10 min (**Figure 2**), resulting in values similar to those observed from the freeze-dried samples. Approximately 98% extraction was obtained within 20 min, close to values observed from the freeze-dried samples. These results suggest that SFE is a reliable method for extracting enrofloxacin from chicken muscle samples. The total volume of methanol consumed to reach almost complete extraction was estimated theoretically to be 12 mL. By using the SFE with optimal extraction parameters, a recovery test of fortified enrofloxacin-free muscles at 50 and 100 ppb was conducted. The recovery percentages ranged from 101 to 104%, with a maximum of 7% standard deviation (**Table 1**). High recovery percentages of enrofloxacin were observed at all levels of concentrations tested, suggesting that our method was consistent with good reproducibility.

Table 1. Recovery Percentage Values of Enrofloxacin from the Fortified Chicken Breast Muscles

enrofloxacin fortified (ppb)	recovery[a] (%)
50	100.6 ± 4.0
100	104.4 ± 6. 7

[a] Values given are the means ± SD of triplicate measurements.

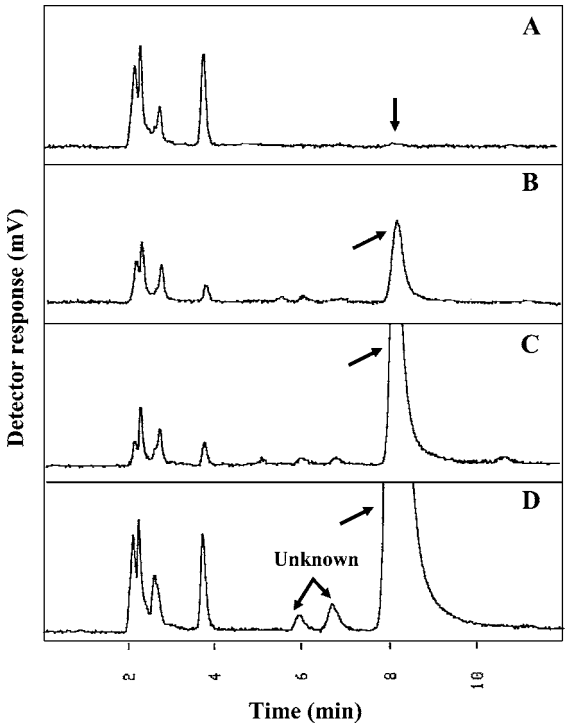


Figure 3. Typical chromatograms of control chicken muscle (**A**), chicken muscles fortified with enrofloxacin at 50 (**B**) and 100 ppb (**C**), and chicken muscle 12 h after oral treatment (**D**). The arrow symbols represent the retention times of the chromatogram peak of enrofloxacin.

Figure 3 shows HPLC chromatograms of the control (**Figure 3A**), enrofloxacin-fortified (**Figure 3B**,**C**), and enrofloxacin-administered (**Figure 3D**) samples that were extracted using the SFE. No detectable chromatogram peak at the retention time of enrofloxacin was observed in the control sample. Supercritical CO_2 is considered to be a nonpolar solvent with a polarity similar to that of hexane, which allows efficient extraction of nonpolar compounds (*23*). In our study, SFE, with supercritical CO_2 plus 20% (v/v) methanol, was found to be suitable for the extraction of enrofloxacin from the chicken breast muscles. SFE, with only supercritical CO_2, gave lower recovery values than supercritical CO_2 plus 20% (v/v) methanol, suggesting that methanol, as a modifier, helped with the extraction. Data taken from the chromatogram of the control sample showed that the lipid matrices extracted with the enrofloxacin did not affect the chromatogram analysis of the control sample. No interference in the peaks of the region near the peak of interest in the control sample suggests that the SFE was efficient in the extraction of enrofloxacin from the muscles.

We examined a time course determination of enrofloxacin in the muscles of orally treated chickens. The muscles were extracted using the SFE at each sampling time after treatment. Enrofloxacin was estimated to be ~918 ± 77 ppb (wet weight basis) 6 h after treatment. The concentration of enrofloxacin in

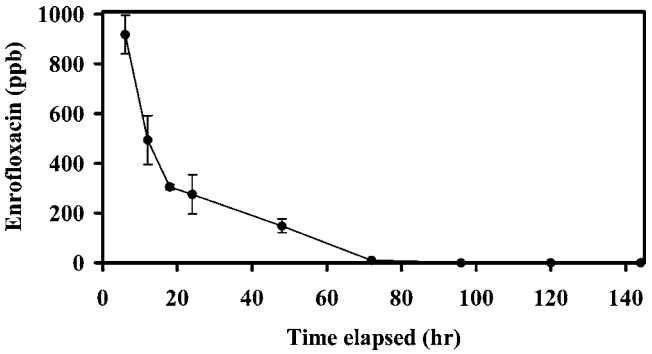


Figure 4. Time course determination of enrofloxacin in the chicken breast muscles treated orally with Enprotil. The data given are the means ± SD of three animals.

the muscles decreased with time, giving a level similar to the LOQ level (5 ppb) 72 h after treatment (**Figure 4**). No detectable enrofloxacin was observed 120 h after treatment, suggesting the possibility that enrofloxacin was depleted completely from the muscles in that time. Two unknown trace peaks were observed from the samples extracted 12 h after treatment (**Figure 3D**). One of them showed the same retention time as that of a ciprofloxacin standard. Ciprofloxacin is a main metabolite of enrofloxacin (*2*, *6*, *11*, *13*, *24*). Enrofloxacin might be degraded to ciprofloxacin in chickens, and both agents may be found in chicken muscles. These results suggest the possibility that the SFE method could extract both enrofloxacin and its metabolite simultaneously. This hypothesis has been already examined in the previous study (*22*), where four fluoroquinolones, including enrofloxacin and ciprofloxacin, were simultaneously extracted using SFE from eggs. More studies, however, are required to examine if the SFE method is applicable in the simultaneous extraction of fluoroquinolones from chickens.

Numerous methods to determine antibacterial agents in biological samples have been reported (*25*, *26*). Recently, a rapid spectrofluorometric method has been documented for more speedy determination of enrofloxacin in chickens (*27*). More sensitive liquid chromatography−mass spectrometry techniques have been well documented (*16*, *17*). Antibacterial agent analysis generally requires the blending samples in either organic or aqueous−organic solvents. Biological samples are problematic in drug analysis, because they have the potential to form interfering compounds during extraction. Consequently, the solvent extraction method requires further experimental steps prior to analysis. For example, solid phase extractions, such as ion exchange, adsorption, or reversed phases, for the isolation of enrofloxacin from biological samples have been reported as a cleanup method (*28*−*30*). Our SFE method is suggested as an alternative to solvent extraction as it does not require cleanup procedures after extraction. Good recovery percentages of fluoroquinolones, similar to conventional methods using solvents, were reported in the previous study (*22*). In the present study, the application of the SFE method was successful for the extraction of enrofloxacin from chicken breast muscles.

ABBREVIATIONS USED

SFE, supercritical fluid extraction; HPLC, high-performance liquid chromatograph; S/N, signal to noise; LOD, limit of detection; LOQ, limit of quantitation.

ACKNOWLEDGMENT

We thank anonymous reviewers for their helpful comments and are indebted to Prof. E. D. Morgan for helping to improve the manuscript.

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Received for review June 18, 2003. Revised manuscript received September 29, 2003. Accepted October 1, 2003. This work was supported financially by the Korea Research Foundation (Grant KRF-2002-041-F00015).

JF0346511