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Liquid Chromatographic Determination of Fluoroquinolones in Egg Albumen and Egg Yolk of Laying Hens Using Fluorometric Detection

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A liquid chromatographic method was developed for the determination of ciprofloxacin, enrofloxacin, and sarafloxacin at 10−200 ppb in both egg yolk and egg albumen of laying hens. Egg yolk or albumen was acidified with 1 M phosphoric acid followed by deproteination with acetonitrile and centrifugation. The supernate was pipetted out, and the remaining protein pellet was extracted three times with acetonitrile. The supernates were combined and concentrated at 50 °C to <0.7 mL. The final volume was adjusted to 2 mL with 0.02 M potassium phosphate buffer, pH 2.5. Separation of the analytes was achieved using reversed-phase HPLC with fluorometric detection. The recoveries were >80% and coefficients of variation <20%. After validation, the method was applied for use in a national survey for fluoroquinolones in table eggs. Of the 276 eggs assayed, none was found positive for fluoroquinolones. The findings suggest that illegal use of fluoroquinolones in laying hens is not widespread.

KEYWORDS: Fluoroquinolones; eggs; HPLC; method; survey

INTRODUCTION

Fluoroquinolones (FQs; **Figure 1**) are antibacterials commonly used in veterinary medicine for the treatment of colibacillosis and other bacterial infections in broiler chickens and turkeys. Among the FQs, sarafloxacin (SARA) was the first approved for use in food-producing animals in the United States (*1*). This was followed by the approval of enrofloxacin (ENRO) for use in poultry for the control of mortality associated with *Escherichia coli* and *Pasteurella multocida* (*2*). In 1997, the US FDA banned the extra-label use of fluoroquinolones because of concerns regarding the development of resistant stains of bacteria (*3*). In April 2001, the FDA withdrew the approvals of two new animal drug applications (NADAs) for SARA, on the basis of new data and information regarding the human food safety of FQ uses in poultry (*4*). The use of SARA and ENRO in laying hens has always been prohibited and illegal in the United States. Illegal extra-label use of FQs may exist and result in violative residues in the human food supply. Analytical methods are therefore needed for monitoring purposes.

The determination of FQs using liquid chromatography (LC) has been described for whole eggs and for egg yolk and egg albumen separately. Maxwell et al. (*5*) developed a method for the determination of SARA in whole eggs using an automated sequential trace enrichment of dialysates (ASTED) system and high-performanc liquid chromatography (HPLC) with fluorescence detection. Schneider and Donoghue (*6*) described a multiresidue method for the determination of six FQs in whole eggs using an ASTED system and HPLC. These methods were developed for whole egg and required the use of a specialized ASTED system. Gorla et al. (*7*) reported a method for the determination of ENRO and ciprofloxacin (CIPRO) in egg yolk and egg albumen using HPLC with ultraviolet detection. However, the reported limits of detection (0.019 μg/g for ENRO and 0.156 μg/g for CIPRO) were high, especially for CIPRO, and the reported recoveries (49−85% for ENRO and 36−50%

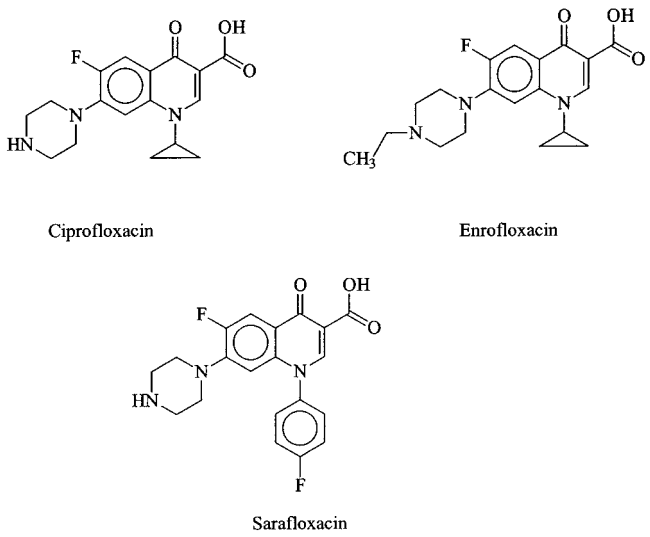

Figure 1. Structures of fluoroquinolones (FQs).

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for CIPRO) were too variable for use in monitoring purposes. Recently, we reported a radiotracer study on [^{14}C] SARA (*8*). In that study, we showed that radioactive residues were transferred into egg yolk and egg albumen after oral administration of [^{14}C]SARA to laying hens and that parent SARA was the predominant residue in both egg yolk and egg albumen. Accordingly, there is a need for analytical methods for monitoring FQs in eggs of laying hens. In this paper, we describe a simple and sensitive HPLC method for the determination of CIPRO, ENRO, and SARA in both egg yolk and egg albumen and report on our results of a national survey for FQs in table eggs.

MATERIALS AND METHODS

Apparatus. The LC system consisted of a Perkin-Elmer series 410 pump, a Perkin-Elmer LS-4 or LC 240 fluorescence detector (Norwalk, CT) interfaced to a PE-Nelson Turbochrom v. 4.1 data system, and a Perkin-Elmer ISS-100 or ISS-200 autosampler equipped with a 200 μL loop. The LC column was a PLRP-S, 5 μm, 4.6 × 150 mm, with guard column of the same packing (Polymer Laboratories Inc., Amherst, MA). A TurboVap LV evaporator (Zymark, Hopkinton, MA) was used for sample concentration. The LC mobile phase was filtered through 0.22 μm nylon-66 (N-66) filters with a solvent filtration apparatus. Prior to injection onto the HPLC, sample extracts were filtered through 13 mm 0.2 μm Acrodisc nylon (Gelman, Ann Arbor, MI) or 22 mm 0.2 μm Millex-GP (Millipore, Bedford MA) filters.

Reagents. LC grade water was purified in-house with a Milli-Q Plus water system and was used in preparing all solutions. Acetonitrile (ACN), phosphoric acid (H_3PO_4, 85%), and trifluoroacetic acid (TFA) were of HPLC grade. SARA-hydrochloride was obtained from Abbott Laboratories (North Chicago, IL); ENRO and CIPRO were obtained from Bayer Corp. (Kansas City, MO). The HPLC mobile phase consisted of ACN/0.1% aqueous TFA (25+75). Its exact composition varied slightly between columns. The preparation of 0.1% aqueous TFA was as follows: 1 mL of TFA was measured into a 1 L volumetric flask. Water was added to the mark. The solution was stirred and filtered through a 0.2 μm nylon filter. The aqueous and organic components of the mobile phase were mixed by the HPLC.

Standard Solutions. *Stock Solutions (100 μg/mL).* On the basis of the listed potency or purity of the standard, the amount of CIPRO, ENRO, and SARA needed to prepare 100 mL of the individual 100 μg/mL standard solutions was calculated. All FQ concentrations are expressed as the free base equivalent. The standard was weighed to the nearest ±0.1 mg into a 100 mL volumetric flask and brought to the mark with water (for SARA) or methanol (for CIPRO and ENRO). These solutions should be used immediately for the preparation of the fortification solution below.

Fortification Solution (2 μg/mL of FQs). Two milliliters of each stock standard solution (100 μg/mL) was pipetted into a 100 mL volumetric flask and brought to the mark with Milli-Q Plus water. This solution may be stored at −80 °C and is stable for at least 12 months.

Calibration Standards. The *fortification solution* (2 μg/mL of FQs) was diluted with 0.02 M potassium phosphate, pH 2.5, to prepare a five-point standard curve of the FQs at the following concentrations: 25, 50, 100, 200, and 400 ng/g (ppb). For levels at or below 20 ppb, a standard curve at 2.5, 5, 10, 20, and 40 ppb was prepared instead. These solutions should be prepared fresh.

Samples. Control eggs were obtained from a local grocery or from our animal facility. Eggs containing incurred residues of CIPRO, ENRO, and SARA were derived from White Leghorn hens individually dosed with 17.9 mg of CIPRO in capsules for two consecutive days, 9.7 mg of ENRO as a liquid by gavage for two consecutive days, or 15 mg of SARA in capsules for five consecutive days. Eggs were collected for at least 10 days after initial dosing. The egg yolk and egg albumen were separated and stored at −80 °C until analysis.

Extraction Procedure. Egg yolk or egg albumen (2.0 ± 0.2 g) was weighed into a disposable 15 mL polypropylene centrifuge tube. The *fortification solution* was added at this step for recovery measurement and gently vortex-mixed. H_3PO_4 (1 M; 0.5 mL) was added to the sample in the centrifuge tube, followed by 2 mL of ACN, and the sample was stirred using a spatula to ensure adequate mixing. The centrifuge tube was capped and vortex-mixed at high speed for 30 s, followed by centrifugation for 5 min at 3800 rpm (3000*g*) at 4 °C to effect phase separation. The clear supernatant was transferred, using a disposable glass pipet, into a separate 15 mL polypropylene centrifuge tube. Two milliliters of ACN was added, and the extraction procedure was repeated three more times. The supernatants were combined and centrifuged for 5 min at 3800 rpm (3000*g*) at 4 °C. The supernatant was transferred into a glass test tube calibrated at 2 mL. Using a TurboVap LV evaporator, the sample was concentrated at 50 °C to <0.7 mL; care was taken not to evaporate to dryness. The final volume was adjusted to 2 mL by adding 0.02 M potassium phosphate buffer, pH 2.5, to the calibrated mark, and the sample was vortex-mixed. The contents were transferred into a 3 cm^3 syringe fitted with a 13 mm Acrodisc nylon or a 22 mm Millex-GP filter and filtered into a glass autosampler vial. Fifty microliters was injected onto the LC system within 2 days after preparation, as we noted an increase in recovery in egg yolk for ENRO stored for 6 days or longer.

Chromatographic Conditions. Egg extracts were analyzed for FQs using the following isocratic LC conditions: mobile phase, 0.1% aqueous TFA/ACN (75+25); flow rate, 1.0 mL/min; excitation wavelength, 280 nm; emission wavelength, 460; run time, 10 min; and column temperature, 35 °C. A water blank was injected to equilibrate the LC system. Fifty microliters of each standard series was injected prior to injecting a sample set. At the end of each day's analyses, the analytical column and guard column should be flushed with water/ACN (50+50, v/v).

Quantitation. A calibration curve of peak area versus concentration (ppb) of each analyte was plotted. Least-squares regression parameters for the calibration curve were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y = peak area and X = concentration of the standard in ppb. Correlation coefficients for each of the calibration curves were routinely >0.99. When unknown or incurred samples are assayed, a control and a fortified control should be processed along with each set for quality control.

National Survey. With the assistance of the Office of Regulatory Affairs (ORA), U.S. Food and Drug Administration, in sample collection, we sampled 276 eggs from 75 egg production or distribution firms throughout the United States over a 9-month period (September 1999 to June 2000). The sampling scheme was devised by CVM statisticians to obtain representative and independent sources of eggs. The 50 U.S. states were subdivided into five geographic regions, based on the available information on the regional variations in shell egg production. Then a total of 75 firms were randomly selected and allocated across these five regions, proportionate to the number of eggs produced in that region. The egg collection was accomplished by ORA field agents, who collected from each firm a dozen eggs of four different egg types, such as jumbo, large, medium, and small and either white or brown. One egg from each egg type was randomly selected and assayed using the above method.

RESULTS

The method was validated using control egg yolk and egg albumen fortified with CIPRO, ENRO, and SARA in the range of 10−200 ppb. Results of these analyses are presented in **Tables 1** and **2**. Recoveries of the three drugs were >80% with CVs <20%. Typical chromatograms of control, fortified control, and incurred egg yolk are shown in **Figure 2**. Corresponding chromatograms of the FQs in egg albumen are shown in **Figure 3**. The method was then applied to samples containing incurred residues of the three FQs. Parent drugs were clearly present in the respective chromatograms of incurred egg yolk and egg albumen and demonstrated that the method can successfully recover the biologically incurred residues. Furthermore, CIPRO was found in the egg yolk and egg albumen of ENRO-treated hens (**Figures 2e** and **3e**), indicating that CIPRO is a metabolite

Table 1. Recovery of Fluoroquinolones from Egg Yolk

		CIPRO		ENRO		SARA	
fortification level (ppb)	*n*	av recovery (%)	CV (%)	av recovery (%)	CV (%)	av recovery (%)	CV (%)
10	6	83	9	106	9	94	4
20	6	86	11	108	11	94	7
50	6	91	5	99	3	95	3
100	6	91	2	100	2	95	2
200	6	91	2	101	2	95	2

Table 2. Recovery of Fluoroquinolones from Egg Albumen

		CIPRO		ENRO		SARA	
fortification level (ppb)	*n*	av recovery (%)	CV (%)	av recovery (%)	CV (%)	av recovery (%)	CV (%)
10	6	87	9	87	13	107	5
20	6	95	6	90	7	102	8
50	6	106	2	91	2	98	10
100	6	103	3	89	3	96	8
200	6	106	4	92	3	100	4

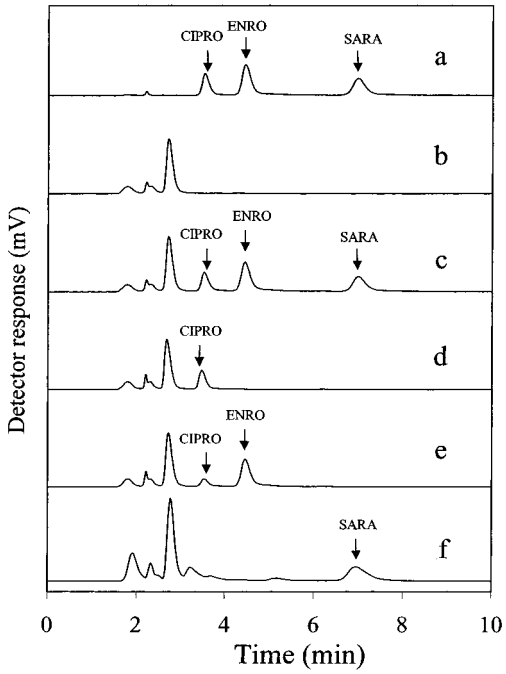

Figure 2. Chromatograms of FQs extracted from egg yolks: (a) 100 ppb mixed standard; (b) control; (c) control fortified with 100 ppb of FQs; (d) incurred CIPRO; (e) incurred ENRO; (f) incurred SARA.

of ENRO. Similar findings have been reported by Gorla et al. (*7*). The levels of incurred residue found are shown in **Table 3**. The limits of detection (LODs) were calculated as defined in the U.S. Pharmacopeia National Formulary (*9*) and estimated to be 1 ppb for all three FQs in egg albumen and 3, 1, and 1 ppb for CIPRO, ENRO, and SARA in egg yolk, respectively.

In the chromatogram of SARA-incurred egg albumen (**Figure 3f**), there was a peak eluting at ~5 min. This peak, however, was not so prominent in the corresponding incurred or fortified egg yolk. It may first appear that this peak is a metabolite of SARA, yet its presence in the fortified egg albumen (see **Figure 3c**) suggested otherwise. Besides, this peak's detector response was directly related to the amount of SARA fortified, suggesting that it is a degradation product formed as a result of SARA and the egg albumen. Although its chemical identity remains unclear,

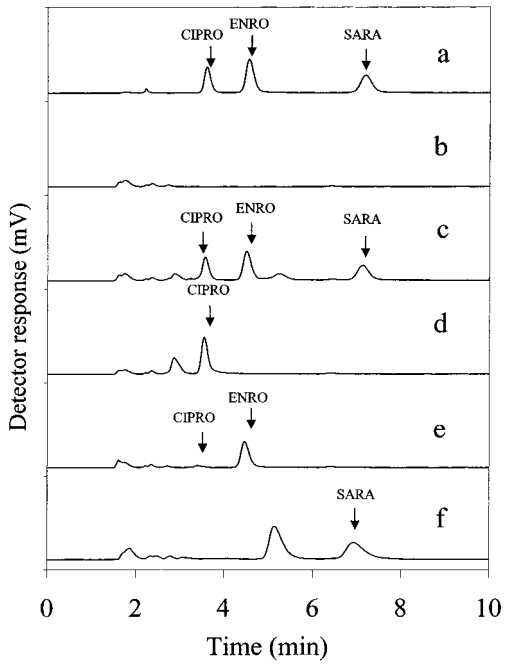

Figure 3. Chromatograms of FQs extracted from egg albumen: (a) 100 ppb mixed standard; (b) control; (c) control fortified with 100 ppb of FQs; (d) incurred CIPRO; (e) incurred ENRO; (f) incurred SARA.

Table 3. Fluoroquinolone Levels Found in Egg Yolk and Egg Albumen of Dosed Hens

			CIPRO		ENRO		SARA	
drug treatment	sample	*n*	ppb found	CV (%)	ppb found	CV (%)	ppb found	CV (%)
CIPRO	yolk, D9[a]	6	73	2				
	albumen, D4	6	156	6				
ENRO	yolk, D9	6	35	4	89	3		
	albumen, D1	6	10	7	71	3		
SARA	yolk, D3	6					80	4
	albumen, D4	6					131	2

[a] D = day of collection after the first dose.

our previous radiotracer study (*8*) combined with the above evidence suggests that it is not a metabolite of SARA.

To demonstrate the selectivity of the method, other approved veterinary drugs including tetracycline, oxytetracycline, chlortetracycline, doxytetracycline, amoxicillin, ampicillin, cloxacillin, penicillin G, penicillin V, cephapirin, ceftiofur, sulfachloropyridazine, sulfamethoxypyridazine, sulfaguanidine, sulfadiazine, sulfamerazine, sulfamethoxazole, sulfamethiazole, sulfisoxazole, sulfathiazole, sulfaquinoxaline, sulfamonomethoxine, sulfadimethoxine, sulfanilamide, sulfaacetamide, sulfamethazine, tylosin, monensin, salinomycin, lasalocid, novobiocin, narasin, ivermectin, and doramectin were chromatographed. None of these drugs interfered with the method.

After validation, the method was applied for use in a national survey. Because our radiotracer study indicated that drug residues remain in the egg yolk for a longer time than in the egg albumen and that egg yolk is a better matrix for monitoring, we conducted our assays on egg yolk only. Of the 276 eggs assayed, none was found positive for FQs. Because it was not possible to estimate a weighted confidence interval with no positive sample, CVM statisticians had to arbitrarily change one result from undetectable to detectable residue and thereby derive an upper bound of the 95% confidence limit of 1.1%.

DISCUSSION

In our previous radiotracer study on [^{14}C]SARA (*8*), we showed that radioactivity was detected in egg yolk and egg albumen on the second day of dosing and that the parent drug was the major component in both egg albumen and egg yolk. The findings suggest a need for a multiresidue method for monitoring FQs in table eggs. In the early stage of our method development, we used a Waters Symmetry C_8 column for chromatographic separation, yet during interference testing, SARA and difloxacin (DIFLOX) were only partially separated. Accordingly, the HPLC conditions were optimized, and separation of the four FQs was achieved with the Polymer PLRP-S column. We also explored the use of a 5 μm, 150 $\times$ 2.1 mm, Inertsil phenyl column, a flow rate of 0.5 mL/min, and a mobile phase consisting of 11% ACN and 89% 0.1% formic acid. This HPLC condition also provided adequate separation and can be used as a substitute. However, the PLRP-S is the preferred column because of its chemical stability at low pH.

During initial method development, the addition of phosphoric acid to the egg samples was not part of the extraction procedure. Subsequent experiments revealed that this step was crucial in recovering FQs. Furthermore, stirring the protein pellet during the ACN deproteination is critical; otherwise, sporadic low recovery may result. This effect is particularly significant for egg yolk, because it forms a harder pellet and traps more analytes than does the egg albumen. To help release the trapped analytes, we incorporated a stirring step with a glass rod or spatula into the method. With these steps, the recovery and precision were markedly improved. In the assay of the yolk extracts, we noticed an increase in recovery for ENRO after prolonged storage. The cause for this effect is not clear; however, it might be related to an increase in matrix interference, which caused fluorescence response enhancement. The extracts therefore should be assayed as soon as possible after preparation.

With the method developed and validated, our next endeavor was to conduct a national survey for FQs in table eggs. The objective of the survey was to identify any illegal use of FQs and to ensure that the U.S. egg supply is safe. None of the samples tested was found positive for FQs. The findings of the survey suggest that the occurrence of FQ residues is $<1.1\%$. The implication of the findings is that illegal use of fluoroquinolones in laying hens is not widespread.

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