Determination of Total ^{14}C Residues of Sarafloxacin in Eggs of Laying Hens

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[^{14}C]Sarafloxacin was orally administered to six laying hens for five consecutive days. Eggs were collected for 15 days after the initial drug treatment. Egg yolk and egg albumen were separated and assayed for total radioactive residues (TRR) using a combustion oxidizer and scintillation counting techniques. Radioactivity was detected in egg yolk and egg albumen on the second day of dosing and reached a maximum at 24 h after drug withdrawal. Thereafter, the sarafloxacin TRR levels in egg albumen declined rapidly and were undetectable 2 days after the last dose, whereas the levels in egg yolk declined at a much slower rate and were undetectable 7 days after drug withdrawal. In both the egg albumen and yolk, HPLC analysis indicated that the parent sarafloxacin was the major component.

Keywords: *Fluoroquinolone; sarafloxacin; residues; egg*

INTRODUCTION

Sarafloxacin (SARA; Figure 1) is a fluoroquinolone antibacterial commonly used in veterinary medicine for the control of early mortality in growing turkeys and broiler chickens associated with *Escherichia coli* organisms (*Code of Federal Regulations*, 1997). Its pharmacological activity is attributable to its inhibition on DNA gyrase (Shen et al., 1989). In the United States, SARA is the first fluoroquinolone approved for use in food-producing animals. Its use in laying hens, however, is illegal. Under the Animal Medicinal Drug Use Clarification Act (AMDUCA), veterinarians under certain conditions are allowed to prescribe certain approved animal drugs and approved human drugs for extra-label use in animals (*Federal Register*, 1997). However, the extra-label use of fluoroquinolones is prohibited by the U.S. Food and Drug Administration because of the growing concern about the development of bacteria resistant to fluoroquinolones (CVM News Article, 1996).

Despite the wealth of methods reported for the determination of SARA in milk (Roybal et al., 1997), animal tissues (Cohen et al. 1999), and aquatic species (Hormazabal et al., 1991; Meinertz et al., 1994; Schilling et al., 1996; Steffenak, et al., 1991; Turnipseed et al., 1998), few are available for SARA in eggs. Maxwell et al. (1999) have recently developed a method for the determination of SARA in whole eggs using an automated sequential trace enrichment of dialysates (ASTED) system and HPLC. Other than this, data, such as total SARA residues and their distribution between egg yolk and egg albumen, are lacking. The objectives of this study were to investigate the transfer of SARA into eggs and to determine the total residue levels and metabolic profiles in both egg yolk and egg albumen. To our knowledge, this is the first radiotracer study reported for SARA in eggs of laying hens.

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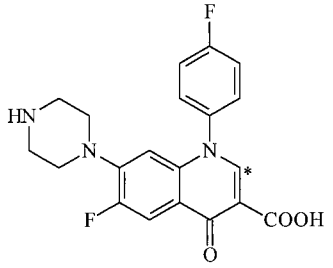

Sarafloxacin

Figure 1. Structure of SARA. The asterisk indicates the carbon-14 position.

MATERIALS AND METHODS

Dose. Non-radiolabeled sarafloxacin hydrochloride (SARA-HCl) was obtained from Abbott Laboratories (North Chicago, IL). [^{14}C]SARA-HCl was synthesized by DuPont NEN (Boston, MA). The dose was prepared by mixing radiolabeled and non-radiolabeled SARA-HCl to attain a specific activity of 13 dpm/ng SARA-base. The radiochemical purity of the dose was determined by HPLC and TLC to be >99%. Water was purified in-house with the Milli-Q Plus water system. Acetonitrile (ACN), trifluoroacetic acid (TFA), tetrahydrofuran (THF), and phosphoric acid (H_3PO_4, 85%) were of HPLC grade.

Animals. Six White Leghorn laying hens housed in individual cages were dosed with 12 mg of [^{14}C]SARA-HCl (equivalent to 10.5 mg of [^{14}C]SARA free base) for five consecutive days. Eggs were collected for analysis through 10 days after the last treatment. After collection, the egg yolk and egg albumen were separated. The egg albumen was homogenized with a polytron (Brinkmann, Westbury, NY), and the yolk mixed with a glass rod. The samples were stored at −80 °C until analysis.

Total Residue Determination. Triplicate yolk samples (~0.3 g) were weighed into combustion boats and assayed for total radioactivity by combustion analysis using a sample oxidizer (Packard model 307, Meriden, CT). The resulting $^{14}CO_2$ was trapped onto Carbosorb (Packard), and radioactivity was measured by liquid scintillation counting (Beckman model LS 5800, Fullerton, CA). The combustion efficiency was 95−97%, as determined by fortifying egg yolk with [^{14}C]SARA (150−4000 dpm) and assayed by combustion analysis. Albu-

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men (~0.5 g) was weighed directly into scintillation vials; 10 mL of Insta-gel (Packard) liquid scintillation fluid was added, and radioactivity was measured by liquid scintillation counting with a count time of 20 min. Total radioactive residue (TRR) concentrations were expressed as sarafloxacin free base equivalents. The limit of detection for TRR in eggs is ~10 ppb. Additional background information on the combustion analysis and scintillation counting techniques is described elsewhere in greater detail (Shaikh and Chu, 2000).

Sample Extraction. Egg yolk or albumen (2.0 ± 0.2 g) was accurately weighed into a polypropylene centrifuge tube. To each sample were added 0.5 mL of 1 M H_3PO_4 and 2 mL of ACN. The sample was stirred with a spatula to ensure mixing followed by vortex-mixing and centrifugation for 5 min at 3800 rpm (3000*g*) at 4 °C. After centrifugation, the clear supernatant was transferred to another 15-mL polypropylene centrifuge tube. The protein pellet was extracted three more times with ACN. The ACN layers were combined and centrifuged for 5 min at 3800 rpm at 4 °C. The supernatant was transferred to a glass tube calibrated at 1 mL and evaporated to <0.7 mL in a heating block at 50 °C. The final volume was adjusted to 1 mL by the addition of 0.02 M potassium phosphate buffer, pH 2.5, to the calibrated mark. The extract was filtered through a 3 cm^3 syringe fitted with a 13-mm, 0.2- or 0.45-μm pore size, disposable nylon filter.

HPLC Analysis. The HPLC system consisted of a Perkin-Elmer Series 4 pump (Norwalk, CT), an Applied Biosystems model 783A UV−vis detector (Foster City, CA) interfaced to a PE-Nelson Turbochrom v. 4.1 data system, and a Perkin-Elmer ISS-100 autosampler equipped with a 200-μL loop. Three HPLC conditions were used for determining the metabolic profiles: (condition 1) HPLC column Symmetry C_8, 5 μm 3.9 × 150 mm, with guard column of the same packing (Waters Chromatography, Milford, MA); mobile phase, 0.1% TFA/ACN (75 + 25, isocratic); flow rate, 1.0 mL/min; detector wavelength, 325 nm; injection volume, 50 μL; (condition 2) HPLC column Alltech Nucleosil C_{18}, 150 × 4.6 mm, with guard column of the same packing (Alltech, Deerfield, IL); isocratic mobile phase: 90% pump A and 10% pump B with pump A ACN/THF/0.02 M potassium phosphate buffer, pH 2.5 (1 + 1 + 18) and pump B ACN/THF/0.02 M potassium phosphate buffer, pH 2.5 (7 + 1 + 12); flow rate, 1.0 mL/min; detector wavelength, 325 nm; injection volume, 50 μL; (condition 3) same as condition 2 above except that gradient elution was used. The gradient was 90% pump A/10% pump B, hold for 10 min, and ramp to 100% pump B in 15 min and hold at final composition for 10 min. The HPLC eluate was fractionated every 20 or 30 s using a Foxy 200 fraction collector (ISCO Inc.). Ultima Gold scintillation fluid (5 mL) was added to each fraction and the radioactivity determined by LSC techniques.

Quantitation of SARA was performed on samples assayed with HPLC condition 1 above and with a UV−vis detector. Typical SARA retention time was ~3.5 min. A five-point calibration curve consisting of 25−400 ppb of non-radiolabeled SARA was used and the SARA concentration interpolated from the curve. The linearity of the UV−vis detector response was >0.99. A control and a 100 ppb fortified control were processed along with each set of incurred samples. The recovery of SARA was ~88%, as calculated using the formula

$$\% \text{ recovery} = (\text{SARA level found}/\text{SARA level fortified}) \times 100\%$$

Mass Spectrometry Structural Confirmation. The confirmation of SARA in incurred egg extracts was performed using a liquid chromatography/multiple-stage mass spectrometry on a LCQ system (Finnigan, San Jose, CA) with a heated capillary electrospray ionization (ESI) interface. A 4.6 × 15 cm PLRP-S column (Polymer Laboratories, Amherst, MA) and a flow of 1 mL/min were used. The mobile phase consisted of ACN and 0.1% formic acid (24 + 76). In the HPLC/MS-MS-MS analysis, the protonated molecular ion (M + 1) for SARA (*m*/*e* 386) was utilized as the precursor ion for collisionally induced dissociation (CID). The product ion (*m*/*e* 342) was stored in the ion trap, utilized as the precursor ion for the next stage of MS, and dissociated through CID to produce a full-scan product ion spectrum. The three product ions (*m*/*e* 322, 299, and 285) were measured, and the 299/322 and 285/322 ion ratios were calculated. The presence of SARA was confirmed when the product ion ratios agreed with those of a reference standard.

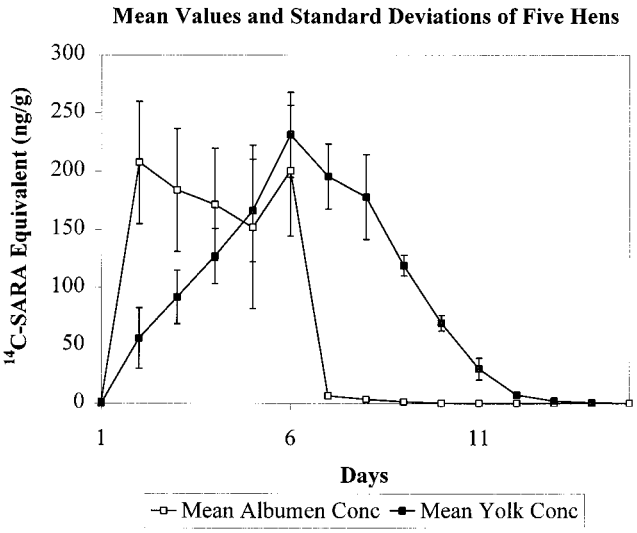

Figure 2. Total radioactive residues of SARA in egg yolk and egg albumen.

Table 1. Distribution of Radioactivity in Egg Albumen between the ACN and Solid Fractions of Hen 3

	ACN/TRR (%)	solid/TRR (%)	total (%)
D2W3[a]	82.3	5.4	87.7
D3W3	80.1	5.5	85.7
D4W3	83.0	6.2	89.2
D5W3	78.8	5.7	84.5
D6W3	79.2	6.0	85.2
av			86.5

[a] D, day postdose; W, egg white.

RESULTS AND DISCUSSION

The mean levels of total radioactive residues (TRR) in egg yolk and albumen of laying hens after oral treatment of [^{14}C]SARA for five consecutive days are shown in Figure 2. Because one hen was not a consistent egg layer, its data were not included in the figure. After drug administration, radioactivity was detected in egg albumen and egg yolk on the second day of dosing. During the dosing period, the mean TRR level in the egg albumen was maintained between 150 and 210 ppb, while the level in the egg yolk rose steadily and peaked at 231 ppb at 24 h after drug withdrawal. Thereafter, the TRR level in egg albumen declined rapidly to <20 ppb within 2 days. There was a marked difference between the slopes of the elimination curves of egg yolk and egg albumen, indicating that they followed different depletion patterns. After drug withdrawal, radioactive residues were detectable for up to 6 days in yolk and for up to 1 day in the albumen.

For both egg yolk and egg albumen, the majority of the radioactivity was extracted into ACN with 5−14% remaining in the protein precipitate (Tables 1 and 2). Accordingly, the ACN extracts were analyzed on HPLC. Three different HPLC conditions were attempted to separate potential metabolites. For all three conditions, the radiochromatograms of both egg yolk and albumen exhibited a single radioactive peak, which was con-

Table 2. Distribution of Radioactivity in Egg Yolk between the ACN and Solid Fractions of Hen 3

	ACN/TRR (%)	solid/TRR (%)	total (%)
D2Y3[a]	73.5	7.6	81.0
D3Y3	81.0	8.6	89.6
D4Y3	80.0	10.3	90.3
D5Y3	74.9	11.3	86.2
D6Y3	73.3	10.6	83.9
D7Y3	73.9	11.3	85.2
D8Y3	86.5	12.5	99.1
D9Y3	76.2	14.0	90.2
D10Y3	70.8	13.0	83.8
av			87.7

[a] D, day postdose; Y, egg yolk.

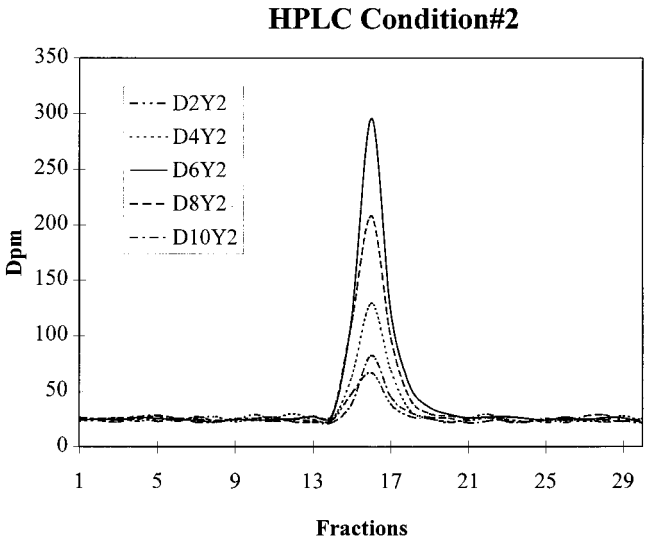

Figure 3. Radiochromatograms of egg yolk of hen 2 treated with [^{14}C]SARA.

firmed as SARA by cochromatography with the authentic standard and by HPLC/MS-MS-MS. Figure 3 shows the radiochromatograms at various time points obtained with HPLC condition 2. The mean percentage of TRR present as SARA was 71% for both egg albumen and egg yolk, indicating that little primary metabolism takes place in laying hens. A similar metabolic profile was reported by Schilling et al. (1996) in the distribution of [^{14}C]SARA in catfish, where the parent SARA was found to be the only radioactive peak detected.

The findings of this study suggest that egg yolk is a more appropriate matrix for monitoring SARA residues than egg albumen, as residues have a longer residence time in the egg yolk than in egg albumen. This is because the majority of yolk formation occurs ~2 weeks prior to ovulation (Johnson, 2000) and these developing yolks serve as a storage depot for drug residues (Donoghue et al., 1997a,b). Furthermore, our findings suggest that the parent SARA is an appropriate marker for monitoring SARA residues.

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