Determination of Sarafloxacin Residues in Fortified and Incurred Eggs Using On-Line Microdialysis and HPLC/Programmable Fluorescence Detection

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Isolation of sarafloxacin (SAR) from fortified and incurred chicken eggs was done by a combination of liquid−liquid extraction and aqueous on-line microdialysis performed on an automated trace enrichment of dialysates (ASTED) system. The ASTED system coupled a sample cleanup procedure with HPLC and programmable fluorescence detection. Overall recoveries of 87−102% for SAR were obtained from samples fortified over a range of 1−100 ng/g. The relative standard deviation values ranged from 22 to 26% for samples fortified between 1 and 5 ng/g and from 2 to 12% for samples fortified between 10 and 100 ng/g. The limits of detection and quantitation were 0.2 and 1 ng/g, respectively. Eggs containing incurred SAR, which were collected over a 3-day dosing period and for 5 consecutive days thereafter, also were analyzed by using this technique. Because the method is automated, 35 samples can be processed within a 24-h period, which enables large data sets to be acquired over a short time period.

Keywords: *ASTED; on-line microdialysis; fluorescence; HPLC; sarafloxacin; chicken; eggs*

INTRODUCTION

Fluoroquinolones (FQs) are broad-spectrum antibiotics used in veterinary medicine in both Europe and the United States for prevention of infectious diseases. The first FQ petitioned for use in food-producing animals in the United States was sarafloxacin (SAR), which was approved in 1995 by the Food and Drug Administration (FDA) for use in day-old chicks and broiler chickens (*Federal Register*, 1995). In recent years, evidence has accumulated supporting the hypothesis that bacterial resistance in humans to antibiotics such as FQs may occur through multiple pathways, as a result of their presence as trace level residues in the nation's food supply (Acar et al., 1993; Acar and Francousal, 1995; Acar and Goldstein, 1997; Endtz, 1991). Because of this concern, the FDA in 1997 prohibited the nonapproved extra-label use of FQ antibiotics in food-producing animals (*Federal Register*, 1997). The presence of trace level FQ residues in eggs may occur as a result of exposure of laying hens to FQs through the extra-label use of FQs or through the consumption of feed unintentionally cross-contaminated during mixing of nonmedicated or mislabeled feed.

The need exists for rapid, sensitive, solvent sparing techniques to monitor for FQs in animal food products such as eggs. Two methods have been reported to date for the isolation and detection of FQs in eggs (Gorla et al., 1997; Schwaiger and Schuch, 1997). Gorla et al. isolated enrofloxacin (ENR) and ciprofloxacin (CIP) from eggs using a multistep, liquid−liquid extraction technique with reported limits of quantitation (LOQ) of 19 ng/g for ENR and 156 ng/g for CIP. Shwaiger and Schuch used a similar liquid−liquid extraction technique for ENR and CIP in eggs. They fortified egg samples with the two FQs at levels of 7, 15, and 30 ng/g, each with recoveries of 98% for ENR and 92% for CIP, whereas the limit of detection (LOD) for each compound was reported as 7 ng/g. However, neither of these FQ antibiotics has been approved in the United States for use in poultry, which unlike SAR decreases the likelihood of their presence as residues in eggs.

On-line microdialysis is a sensitive, rapid, solvent-sparing technique incorporating high sample throughput that offers several advantages for the analysis of trace level residues in food products. This relatively recent analytical method employs the novel automated sequential trace enrichment of dialysates (ASTED) instrument for sample cleanup as part of the sample preparation step prior to chromatographic analysis. Typically, biological samples are first transferred into an aqueous medium and then automatically injected into a dialysis block, housing a dialysis membrane for removal of unwanted coextracted material. The eluate from the dialysis sequence is then concentrated on a small sorbent bed contained in a trace enrichment column and in the last step injected directly onto an high-performance liquid chromatography (HPLC) column for separation, detection, and quantification (Figure 1).

Although the ASTED technology has not previously been applied to FQs in eggs, its use has been reported for the isolation of FQs and other drug classes from various biological matrices. Examples of its application are the analysis of sulfonamides and selected other drugs in egg, meat, and milk (Aerts et al., 1988, 1991, 1995), anticonvulsants in plasma (Turnell and Cooper,

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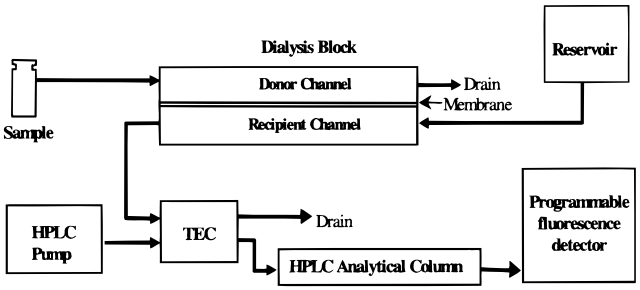

Figure 1. Schematic diagram of the ASTED-HPLC flow pathways.

1987), uracil and its metabolites in plasma (Buick and Fook, 1993), and flumequine and oxolinic acid from fish tissues (Andresen and Rasmussen, 1990; Thanh et al., 1990).

In our initial studies using this technology, we broadened its applicability by developing a procedure for the isolation of flumequine and oxolinic acid from complex and difficult to extract biological matrices, such as chicken liver (Eng et al., 1998). We next reported a technique for greatly increasing the overall sensitivity and multiresidue capability of this technique by combining the ASTED/HPLC system with a programmable wavelength fluorescence detector (PFD) (Maxwell and Cohen, 1998). In the present study we applied the ASTED technology to the recovery and quantification of SAR in both fortified and incurred eggs to develop a solvent-sparing, automated method for this drug at concentration levels of interest to regulatory agencies.

MATERIALS AND METHODS

Sarafloxacin hydrochloride (SAR) (88.5%) was obtained from Abbott Laboratories (North Chicago, IL). Anhydrous diethyl ether, ammonium hydroxide, sodium hydroxide, sodium dibasic phosphate, sodium monobasic phosphate, and Triton X-100 were obtained from Sigma (St. Louis, MO). Triethylamine and phosphoric acid 85% were obtained from Fisher Scientific (Fair Lawn, NJ). A Barnstead NANOpure filter and deionizer unit (Dubuque, IA) provided Nanopure water. Acetonitrile, hexane, and methanol were HPLC grade solvents from Burdick & Jackson (Muskegon, MI). All buffered and nonbuffered solvents and solutions prepared for this study were filtered through a 0.2 μm pore filter prior to use.

FQ-free eggs (control samples) and eggs containing incurred SAR were obtained from FDA/Center for Veterinary Medicine (Laurel, MD). Eleven Single Comb White Leghorn (SCWL) laying hens, in their 50th week of lay, were used in this study. Hens had ad libitum access to standard laying hen feed and water and were subjected to 14 h of light daily. Hens were maintained in normothermic conditions and were individually caged. Seven hens received daily intramuscular injections with 25 mg of SAR for 3 consecutive days. Four hens served as controls for egg collection. Eggs were collected twice daily until 5 days after drug withdrawal. After collection, individual whole eggs were homogenized and stored at −80 °C until needed.

Prior to fortification, control egg samples were thawed for 4 h at 4 °C. These samples were volumetrically divided into 1 mL portions, which were measured into 50-mL polypropylene centrifuge tubes. A stock (25 μg/mL) SAR fortification solution was prepared by dissolving 14.1 mg of SAR in 50 mL of a 0.03 M sodium hydroxide solution and diluting this solution to 500 mL with HPLC grade water, which then was stored at 4 °C in an amber glass bottle. This solution was stable for at least 3 months (Walker et al., 1996). A 1000 ng/mL SAR standard solution was prepared daily by diluting the stock solution with HPLC grade water. Fortification solutions were prepared as needed by diluting the working standard with a 0.1 M sodium phosphate (pH 9.0) buffer solution to final concentrations of 1000, 500, 250, 100, 50, and 10 ng/mL. Fortification of samples in triplicate, at six concentration levels, was performed by delivering a 100 μL aliquot of each fortification solution onto the surface of the homogenized egg contained in the centrifuge tubes. The tubes were then vigorously shaken for 5 min at 25 °C using a Janke & Kunkel IKA Vibrax VXR shaker (Staufen, Germany) and incubated at 4 °C overnight to allow for drug permeation into the egg matrix.

The fortified and incurred egg samples were analyzed in the following abbreviated sequence after debinding and defatting: an initial tissue debinding and defatting step by liquid−liquid extraction was performed followed by sample cleanup using on-line microdialysis (ASTED) and then direct separation, detection, and quantification using HPLC with PFD.

The protein debinding and sample defatting operations in the first step of the above sequence were a modification of a method reported by Hormazabal et al. (1991). The samples were first mixed with 1 mL of acetonitrile on a vortex mixer followed by the addition of 1 mL of a 1.0 M NaCl solution. This mixture was centifuged at 3000*g* and decanted into a second centrifuge tube. A second 1 mL portion of acetonitrile was added to the pellet remaining in the first tube, which then was centrifuged, decanted, and combined with the solution in the second tube. Next, 1.0 mL of hexane and 0.5 mL of ethanol were added to this solution, shaken on a vortex mixer, and centrifuged. The upper organic layers then were carefully removed, and the lower aqueous layer was transferred to a vial for dialysis. Subsequent sample cleanup was performed by on-line microdialysis using an ASTED XL system (Gilson Medical Electronics, Villiers-leBel, France). This system was composed in part of a flatbed dialysis block with donor (370 μL) and recipient (650 μL) channels, separated by a cellulose dialysis membrane (Cuprophan) having a molecular mass cutoff of 15 kDa. Concentration of the SAR eluate following dilution in the dialysis step described below was done by trapping the compound on a custom-made trace enrichment column (TEC) (5.8 mm × 4.6 mm i.d., with 5 μm frits, containing 70 mg of a 10 μm Hypersil ODS sorbent; Keystone Scientific, Inc., Bellefonte, PA). The TEC was connected to the recipient channel of the dialysis block through a model 7010 six-port valve (Rheodyne, Berkeley, CA). Switching this valve connected the TEC to the HPLC system (Rainin Instrument Co., Inc., Woburn, MA). The dialysis membrane and the TEC required replacement only after ∼100 injections. A schematic diagram of the ASTED/HPLC system is shown in Figure 1.

The dialysis sequence was performed in the following manner: A 370 μL sample of the extract was transferred onto the upper portion of the dialysis block at a rate of 0.3 mL/min and programmed to remain stationary in the donor channel for 5 min, after which time three pulses of 650 μL each of 20 mM sodium phosphate (pH 5.0) buffer were pumped at a rate of 0.6 mL/min through the recipient channel and were then concentrated on the TEC. After this step, a second 370 μL sample aliquot was delivered onto the donor channel and similarly dialyzed against three additional pulses of sodium phosphate buffer and concentrated on the TEC. The TEC then was flushed with 500 μL of the same sodium phosphate buffer, and the membrane was washed by rinsing the donor channel with 2 mL of 0.01% Triton X-100 in 20 mM (pH 5.0) sodium phosphate buffer in preparation for injection of the next sample.

The HPLC mobile phase was formed from (a) 0.025 M phosphoric acid buffer solution adjusted with triethylamine (TEA) to pH 2.7 and (b) acetonitrile. The initial mobile phase composition was 65:35 (v/v) buffer/acetonitrile, which was programmed over 9 min to reach a final composition of 42:58 (v/v). This mobile phase composition was held for 1 min, returned over a 5 min interval to its initial composition, and then held for 3 min until the next sample injection. After column switching, the initial mobile phase carried the SAR in a narrow band from the TEC onto a Supelcosil-ABZ + Plus column (5 μm, 250 mm × 4.6 mm i.d., Supelco, Inc., Bellefonte, PA) at a flow rate of 1 mL/min. The Rainin HPLC system was controlled by Rainin Dynamax system software version 1.3, which also processed the data from the Jasco FP-920 PFD

Table 1. Recovery of SAR from Whole Chicken Eggs Fortified at Six Concentration Levels

fortification level,[a] ng/g	recovery,[b] %	RSD %
100	94	3
50	90	2
25	96	12
10	96	10
5	87	22
1	102	26

[a] $n = 3$. [b] Determined from external standard curve responses (peak areas).

(Jasco International Co., Ltd., Easton, MD). The excitation/emission maxima wavelengths were program set at excitation 278 nm, emission 440 nm, standard response, gain 1, and attenuation 256. Quantification of SAR was performed using an external standard curve established from SAR concentrations of 1, 2.5, 10, 25, 50, 100, 250, and 500 ng/g.

RESULTS AND DISCUSSION

The complex nature of egg samples does not permit direct analysis with the ASTED automated sample tray platform, as is possible with less complex matrices such as plasma and milk. For eggs, the debinding and defatting operations must be carried out off-line prior to the final aqueous extracts being loaded onto the sample tray because the ASTED system performs only automated cleanup of aqueous sample extracts. The sample is loaded into the donor channel, which is contained in the upper half of the microdialysis block. A membrane sandwiched between the two sections of the dialysis block (Figure 1) separates the donor channel from the recipient channel located in the bottom half of the dialysis block. Only low molecular mass compounds (<15 kDa) migrate from the donor channel through the membrane into the recipient channel; proteins and other debris remain in the donor channel mobile phase. The analytes of interest, diluted in the dialysis step, then are concentrated on a TEC, which in turn is connected to the HPLC system through a switching valve for analyte detection and analysis.

One advantage of the ASTED system over conventional dialysis techniques is its ability to maintain a steep concentration gradient across the dialysis membrane when operating in the static donor channel−pulsed recipient channel mode, a technique used in the present study. In that mode, the sample is held statically in the donor channel while a predetermined volume of buffer solution is pulsed through the recipient channel. With each pulse dialysis equilibrium is reestablished and thus a more complete migration of the target analytes from the donor to the recipient channel is achieved.

The ASTED/HPLC/PFD system was used to construct standard curves for SAR over a concentration range of 1−500 ng/g. The curves were linear over this concentration range ($R^2 = 0.9989$) with an LOD of 0.2 ng/g and an LOQ of 1 ng/g. We earlier found that this level of sensitivity for SAR analysis is not possible using a single wavelength fluorescence detector in conjunction with the ASTED system (Maxwell and Cohen, 1998).

Egg samples fortified with SAR at six concentration levels were analyzed by using the combined ASTED/HPLC/PFD system (Table 1). Recoveries of SAR over the range of 1−100 ng/g were 87−102%. Relative standard deviation (RSD) values ranged from 22 to 26% for samples fortified at the 1−5 ng/g levels and from 2 to 12% for samples fortified at the 10−100 ng/g levels.

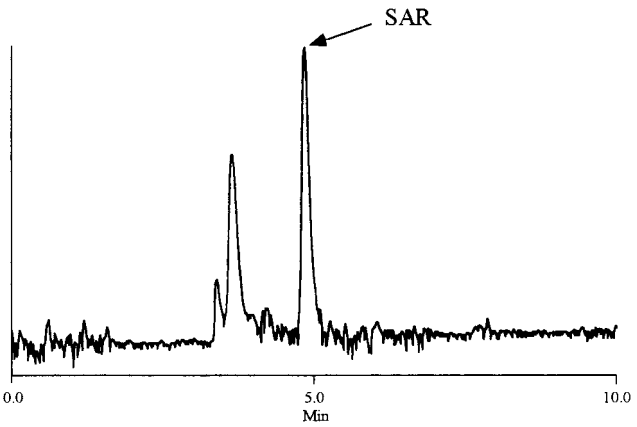

Figure 2. ASTED/HPLC/PFD chromatogram of a control egg sample fortified with 2.5 ng/g of SAR.

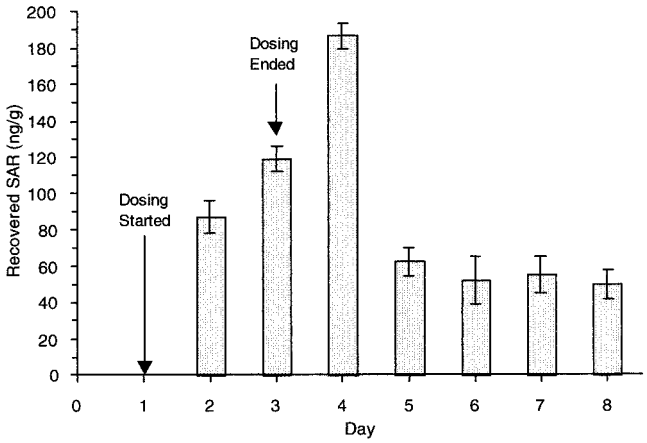

Figure 3. Three-day dosing and five-day withdrawal bar graph for SAR isolated from incurred whole chicken eggs.

Although the RSDs for the 1−5 ng/g samples are high, they are considered acceptable for samples fortified at these low levels (Horwitz, 1980). A typical chromatogram for an egg sample fortified with SAR is shown in Figure 2. In this instance, the sample was fortified with 2.5 ng/g of SAR, which is near the LOQ for this analyte. Although the signal-to-noise ratio was higher than that observed for samples fortified at higher analyte concentrations, the peak for SAR is symmetrical and was easily integrated with respect to the background signal.

We next analyzed for SAR in incurred whole eggs. These data were used to graphically construct the dosing and withdrawal histogram depicted in Figure 3. The data shown in this figure are a summary of a broader set of data (147 chromatograms) obtained from 49 incurred egg samples that were analyzed in triplicate. Error bars represent the RSD for each daily sample set, which are related to the differences in residue concentrations in eggs collected from individual laying hens on a specific day. These values ranged from 7 to 13% over the 8-day dosing and withdrawal period.

Incurred eggs were from chickens dosed with SAR over a 3-day period. Day 1 in Figure 2 is the first day SAR was administered; egg collection was started on the second day. The SAR concentrations reached their highest levels (119 ng/g) 1 day after the last injection (day 4). Thereafter, the SAR concentration fell to, and leveled off at, an average concentration of 50 ng/g over the remaining 4 days. This dosing and withdrawal

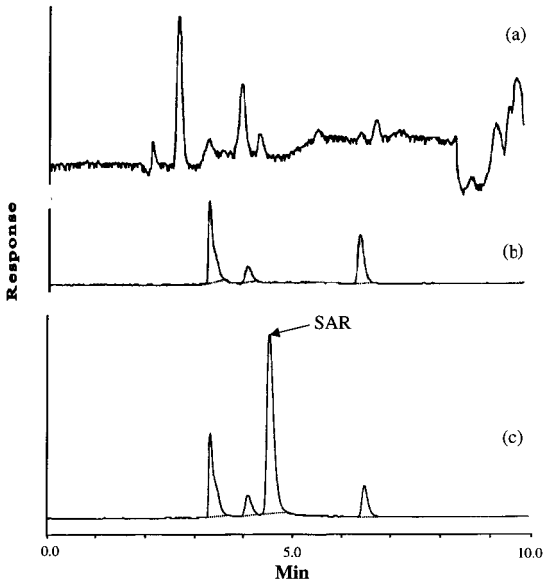

Figure 4. ASTED/HPLC/PFD chromatograms of (a) control sample extract prior to ASTED cleanup, (b) control sample extract after ASTED cleanup, and (c) SAR incurred egg extract after ASTED cleanup at day 5 of withdrawal (day 8; Table 1).

profile is similar to previously reported profiles obtained for other classes of drug residues in eggs (Donoghue et al., 1997).

It was stated previously that an important advantage of on-line microdialysis is its ability to generate chromatograms free of background interference, unlike those often obtained after some conventional sample isolation and cleanup procedures. This feature of the ASTED system is further illustrated by the three chromatograms shown in Figure 4. Figure 4a is a chromatogram of a control egg extract after the sample debinding and defatting sequence but prior to ASTED cleanup. The poor quality of this chromatography may be compared with that from a similar extract shown in Figure 4b. Here the sample was subjected to an ASTED cleanup step. Although the chromatogram in Figure 4b does contain some minor peaks for compounds that migrated through the dialysis membrane, the chromatogram is free of background interference of the type shown in Figure 4a. The benefits of this dialysis cleanup and enrichment technique are further illustrated by the chromatogram shown in Figure 4c of an incurred egg extract containing SAR collected on day 8 of this study. The peak for SAR appears in an elution window that is free of potentially interfering peaks (see Figure 4b), allowing it to be accurately quantified.

CONCLUSIONS

An automated on-line microdialysis method is described for the analysis of SAR residues in fortified and incurred whole eggs. The proposed method is very sensitive and reproducible due to the ability of the dialysis membrane to efficiently separate target analyte(s) from interfering material in the sample matrix. Because microdialysis is essentially an aqueous technique, organic solvent use is minimized, requiring <4.0 mL of solvent/egg sample, an amount that is used only in the initial protein debinding and defatting operations. Because the method is automated, 35 samples can be processed within a 24-h period, enabling large data sets to be rapidly acquired. In addition, the dialysis membrane and the TEC may be used repeatedly, which considerably reduces the cost of consumables. These features make the ASTED system a useful technology for regulatory applications for which automated, rapid, reproducible, and cost-effective methods are needed to screen large numbers of samples for potential violative levels of pharmaceutical residues.

ABBREVIATIONS USED

ASTED, automated sequential trace enrichment of dialysates; SAR, sarafloxacin; TEC, trace enrichment column; PFD, programmable fluorescence detection; FQ, fluoroquinolones; ENR, enrofloxacin; CIP, ciprofloxacin; LOQ, limit of quantitation; LOD, limit of detection; HPLC, hjigh-performance liquid chromagtography; TEA, triethylamine; RSD, relative standard deviation.

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