Immunosorbents Coupled On-Line with Liquid Chromatography for the Determination of Fluoroquinolones in Chicken Liver

Carol K. Holtzapple,* Sandra A. Buckley, and Larry H. Stanker

Food Animal Protection Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, Texas 77845

Four fluoroquinolones were analyzed in fortified chicken liver using an automated, on-line immunoaffinity extraction method. The fluoroquinolones were extracted from the liver matrix using an immunoaffinity capture column containing anti-sarafloxacin antibodies covalently cross-linked to protein G. After interfering liver matrix components had been washed away, the captured fluoroquinolones were automatically eluted directly onto a reversed phase column. Liquid chromatographic analyses were performed by isocratic elution using 2% acetic acid/acetonitrile (85:15) as the mobile phase and an Inertsil phenyl column with fluorescence detection at excitation and emission wavelengths of 280 and 444 nm, respectively. No significant interferences from the sample matrix were observed, indicating good selectivity with the immunoaffinity column. Overall recoveries from fortified liver samples (20, 50, and 100 ng/g) ranged between 85.7 and 93.5% with standard deviations of <5%. The limit of quantification for each fluoroquinolone was 1 ng/mL. The limits of detection, based on a signal-to-noise ratio of 5:1, were 0.47, 0.32, 0.87, and 0.53 ng/mL for ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin, respectively.

Keywords: *Fluoroquinolone; immunoaffinity chromatography; on-line detection method; bovine tissues; food safety*

INTRODUCTION

The fluoroquinolones are antibiotics that are used in both human and animal medicine to treat bacterial infections. Although they have not been traditionally used for food animals because of concern about the possible development of resistant pathogens to this valuable class of drugs, the U.S. Food and Drug Administration (FDA) recently approved sarafloxacin for use in day-old broiler chicks. Other fluoroquinolones are also under consideration for approval. To preserve the effectiveness of these important antibiotics, rapid detection methods are necessary to screen large numbers of samples. Conventional methods (Gau et al., 1985; Morton et al., 1986; Waggoner and Bowman, 1987; Lynch et al., 1994; Roybal et al., 1997) for detecting fluoroquinolones require extensive sample cleanup procedures and cannot be used for routine screening.

Immunoassays have been used as analytical tools for residue analysis in foods and environmental samples. We recently developed a monoclonal antibody-based immunoassay to detect fluoroquinolone residues in chicken liver (Holtzapple et al., 1997). As with similar assays for other types of residues, this assay can be incorporated into monitoring programs to rapidly screen large numbers of samples and eliminate samples that do not contain violative levels of residues. Despite their advantages over traditional chemical methods of analysis (HPLC), immunoassays suffer from the disadvantage that, in many cases, cross-reactivity with structurally related compounds can interfere with the results. Therefore, samples that test positive in the immunoassay must be subjected to conventional chemical methods to identify which residues are present.

To circumvent the cross-reactivity difficulties associated with immunoassays, and to bypass the lengthy sample cleanup procedures associated with HPLC analysis, antibodies have been coupled to immunoaffinity solid phase extraction columns and used to prepare samples for HPLC analysis (Studer-Rohr et al., 1995; Tuinstra et al., 1993; Vanderlaan et al., 1993). More recently, these columns have been incorporated into automated on-line methods that take advantage of the strengths of both immunochemistry- and liquid chromatography-based separation strategies (Newkirk et al., 1998; Nedved et al., 1996). In these methods, an on-line immunoaffinity column is used to "capture" residues of interest while simultaneously allowing components of the sample matrix to be eluted to waste. The residues are then eluted to a restricted access media (RAM) column to decouple the solution conditions used for the immunoaffinity column (aqueous, physiological pH, high salt) from the mobile phase that is optimal for the analytical column (organic solvents, low pH, low salt). The captured compounds are then delivered to the third (HPLC) column for final separation prior to detection.

Here we report detection of fluoroquinolones in chicken liver by automated immunoaffinity capture coupled on-line with HPLC analysis. Alteration of the binding and elution conditions allowed direct delivery of the captured compounds to the second (HPLC) column without the need for a RAM column, thus eliminating an additional step in the method. We observed that the fluoroquinolones elute from the immunoaffinity column on the basis of their relative affinities for the antibody, and the possible applications of this phenomenon to residue analysis will be addressed. Due to the minimal cleanup

* Author to whom correspondence should be addressed [e-mail holtzapple@ffsru.tamu.edu; fax (409) 260-9332].

10.1021/jf990200y
Published on Web 06/25/1999

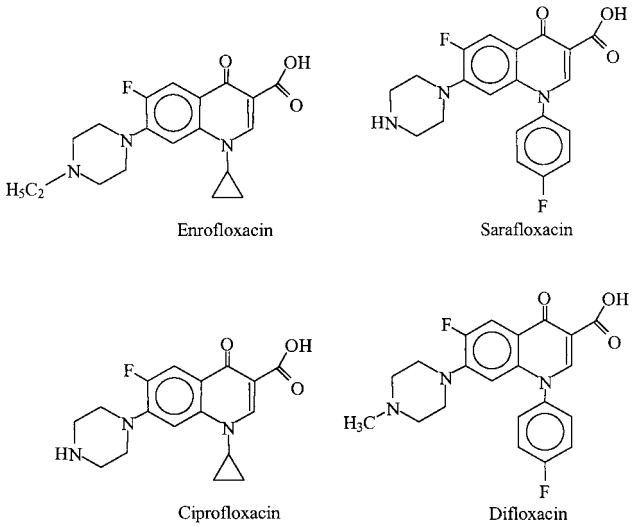

Figure 1. Structures of the fluoroquinolones evaluated in this study.

requirements necessary for the immunoaffinity column and the compound separating capabilities of the HPLC column, this method can be used as a sensitive, multiresidue, on-line method to detect fluoroquinolones in chicken liver.

MATERIALS AND METHODS

Fluoroquinolones. Sarafloxacin and difloxacin (Abbott Laboratories, North Chicago, IL) and enrofloxacin and ciprofloxacin (Bayer; Kansas City, MO) were gifts from their respective manufacturers. The structures of these fluoroquinolones are given in Figure 1.

Purification of Anti-Sarafloxacin Antibodies. Monoclonal antibody Sara-95 (Holtzapple et al., 1997) was purified from ascites fluid using a protein G column (Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, the lipids in 2.5 mL of ascites fluid were removed by combining 1 part of ascites fluid with 1.5 parts of seroclear (Calbiochem-Novabiochem Corp., La Jolla, CA), vortex mixing for 1 min, and centrifuging at 3000*g* for 10 min. The top layer was filtered through a 0.22 μm filter to remove particulate material, mixed with 2.5 mL of column buffer (0.2 M sodium phosphate buffer, pH 7.2), and loaded onto a protein G column equilibrated with column buffer. After unbound proteins had been eluted, the bound antibodies were eluted with elution buffer (0.1 M glycine−HCl, pH 2.7). The antibody fraction was neutralized with 2 M Tris base and then dialyzed in phosphate-buffered saline (PBS; 100 mM sodium phosphate, 150 mM NaCl, pH 7.0). The purified antibodies were stored at −20 °C prior to use.

Immunoaffinity (IAC) and Reversed Phase (RP) Chromatography. An Integral Microanalytical workstation from PE Biosystems (Framingham, MA) was used to perform automated column switching. The system consists of an autosampler, two HPLC pumps, three 10-port switching valves, two reagent syringe pumps, a fluorescence detector, and a variable-wavelength UV detector. The IAC and RP (Inertsil, phenyl, 5 μm, 150 × 4.6 mm; Alltech, Deerfield, IL) columns were linked as shown in Figure 2. Pump 1 delivered the IAC column binding buffers (PBS and 0.1 M NaH_2PO_4, pH 6.0) whereas pump 2 delivered [2% (v/v) acetic acid, pH 2.5] and the RP column mobile phase [85:15 elution buffer/acetonitrile (v/v)]. Capture of the compounds on the IAC column was performed at room temperature, and separation on the RP column was accomplished at 40 °C using a Golden Foil column heater (Alltech). For fluorescence detection, the excitation and emission wavelengths were set at 280 and 444 nm, respectively.

The protein G−purified Mab Sara-95 was brought to a final concentration of 2 mg/mL prior to linkage to the IAC column matrix. The IAC column was made by packing a PEEK cartridge (2.1 mm diameter × 30 mm length) with POROS medium (PE Biosystems, Framingham, MA) containing protein G covalently bound to its surface. Sara-95 was adsorbed to the protein G surface and then covalently cross-linked in place. Briefly, the column was equilibrated with loading buffer (150 mM NaCl, 10 mM phosphate, pH 7.4), and then 1.5 mL of purified Sara-95 (2 mg/mL) was injected through a 2-mL sample loop onto the column using the reagent syringe pump. The cross-linking solution (30 mM dimethyl pimelimidate in 100 mM triethanolamine, pH 8.5) was loaded into the 2-mL sample loop and injected onto the column using the reagent syringe pump. Cross-linking solution was repeatedly injected for a total volume of 14 mL, and then unreacted functional groups on the cross-linking reagent were capped using 2-mL injections of quenching solution (100 mM monoethanolamine, pH 9.0). After the column was cycled between the loading and HCl elution buffers (12 mM HCl, 150 mM NaCl, pH ∼2.0) three times, the column was stored at 4 °C in PBS/0.02% sodium azide to prevent microbial contamination.

Column Switching Events. The column switching capabilities of the Integral system were exploited to capture the fluoroquinolones on the IAC column, elute the bound fluoroquinolones from the IAC column to the RP column, and finally separate the compounds on the RP column prior to fluorescence detection. The plumbing diagram for the system is shown in Figure 2. In the first step (Figure 2A), the IAC column was equilibrated with PBS for 1.5 min at 3 mL/min, and then the fluoroquinolone standards or fortified samples were injected at 0.5 mL/min onto the IAC column using the autosampler and a 100-μL sample loop. The column was washed with PBS at 0.5 mL/min with 10 column volumes (col vol) and then at 2.0 mL/min with 30 col vol. To reduce the amount of salt that would elute to the RP column, the IAC column was washed at 2.0 mL/min with 10 col vol of 0.1 M sodium phosphate buffer (pH 6.0). Previous studies demonstrated that this low-salt, low-pH buffer did not adversely affect antibody binding (Holtzapple and Stanker, 1998). The fluoroquinolones were retained on the column, while matrix components of the samples were flushed to waste.

Just prior to elution of the fluoroquinolones from the IAC column, the RP column was switched in-line with the first column (Figure 2B). The fluoroquinolones were eluted from the IAC column to the RP column with elution buffer (2% acetic acid, pH 2.2) at 0.5 mL/min. After elution, the RP column was switched off-line and the IAC column was equilibrated with 30 col vol (∼3 mL) of PBS at 4 mL/min (Figure 2A).

Both columns were switched off-line so that the lines could be purged with the mobile phase required by the RP column [elution buffer/acetonitrile (85:15 v/v)]. In the final step, the RP column alone was switched in-line (Figure 2C), and the fluoroquinolones that eluted from the IAC column were separated isocratically prior to fluorescence detection using the RP mobile phase at a rate of 0.7 mL/min.

Liver Extract Preparation. Minced samples (1 g) of fluoroquinolone-free chicken liver were fortified with ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin at concentrations of 20, 50, or 100 ppb (ng/g). The fortified samples were suspended in 3.5 mL of 0.1 M NaOH, vortex mixed for 1 min, neutralized with 200 μL of 1 M phosphoric acid, and diluted with 5.5 mL of PBS containing 10% methanol. After centrifugation at 14000*g* for 30 min at 4 °C, the supernatants were passed through a 0.2-μm filter directly into autosampler vials for HPLC analysis. Due to the 10-fold dilution of the liver tissue, the final concentrations of the fluoroquinolones were 2, 5, or 10 ng/mL. Therefore, the 100-μL injection volumes contained 0.2, 0.5, or 1.0 ng/mL of each fluoroquinolone and represent 10 mg of tissue.

RESULTS AND DISCUSSION

Although antibody-based tests have great sensitivity and have been used successfully to rapidly screen food

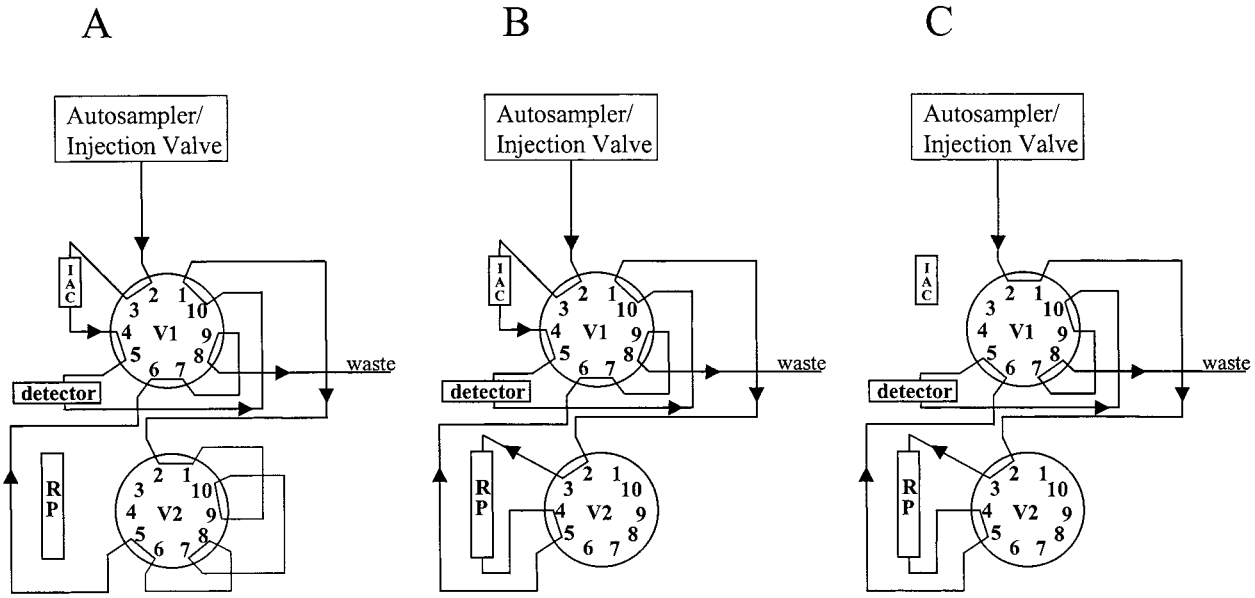

Figure 2. Plumbing diagrams of the dual column (gradient) configuration for the Integral Microanalytical workstation: direction of flow for (A) capture of the analytes in aqueous buffer on a 100-μL IAC column and removal of contaminating materials in the matrix; (B) elution of the captured fluoroquinolones to the RP column; (A) regeneration of the IAC column; (C) separation of the fluoroquinolones on the RP column with fluorescence detection.

products for the presence of unwanted contaminants, their inability to distinguish between structural analogues and their tendency to overestimate the levels of target drugs due to matrix effects limit their usefulness as analytical assays. As a consequence of cross-reacting compounds, the results obtained for a particular sample must be viewed in terms of "equivalents" (Newkirk et al., 1998). In the case of the anti-sarafloxacin antibody, Mab Sara-95 (which recognizes at least six fluoroquinolones), the ELISA results actually reflect "sarafloxacin equivalents". Unfortunately, because the fluoroquinolones exhibit widely different IC_{50} values for the antibody, it is not evident whether the results reflect the presence of sarafloxacin alone, a mixture of fluoroquinolones, or simply interferences from the sample matrix that inhibit binding.

A number of liquid chromatographic methods have been developed to detect the presence of fluoroquinolone residues in a variety of matrixes. Although each fluoroquinolone can be unambiguously identified, these methods require a laborious sample preparation procedure to remove proteins and other matrix components that may interfere with analyses.

To circumvent the disadvantages associated with the cross-reactivity associated with ELISAs and the time-consuming sample preparations required for HPLC analyses, an anti-sarafloxacin monoclonal antibody was used to develop an immunoaffinity column capable of on-line sample cleanup in tandem with analytical HPLC. Using this system, fluoroquinolones are selectively retained on the IAC column and contaminating matrix components are eluted to waste prior to delivery of the fluoroquinolones to the RP analytical column for final analysis.

Two aspects of the method that had to be addressed involved (1) extracting all four fluoroquinolones from the matrix with high recoveries and (2) minimizing the amount of matrix components binding nonspecifically to the IAC column. Because previous ELISA studies (Holtzapple et al., 1997) had demonstrated that sarafloxacin could be effectively extracted from liver using assay buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20, 1% BSA, pH 7.5), the fortified liver samples in this study were initially extracted in this buffer. However, upon analysis, it was evident that enrofloxacin was selectively bound to matrix components, resulting in poor recovery (60%) of this drug despite good recoveries (~85−90%) of the other three fluoroquinolones (data not shown). The addition of up to 20% methanol and/or 0.1% Tween 20 did not significantly improve recovery of enrofloxacin. Because we wanted to develop a multiresidue detection method, the extraction method was changed to recover enrofloxacin at acceptable levels.

Previously published sample preparation methods for HPLC analysis of the fluoroquinolones involved acids or bases followed by solvent extraction (Horie et al., 1994; Hormazabal and Yndestad, 1994; Kaartinen et al., 1995; Tarbin et al., 1992; Tyczkowska et al., 1994). In an effort to effectively extract all of the fluoroquinolones, the tissues in this study were extracted in 0.1 M NaOH and then neutralized with the addition of 1 M H_3PO_4 and PBS (pH 7.2). After a single centrifugation step, the supernatants were syringe-filtered directly into autosampler vials and analyzed. Using this extraction method, the four fluoroquinolones were recovered at levels >85%.

Once an acceptable extraction process was determined, the amount of sample matrix that bound nonspecifically to the IAC column had to be minimized. As was observed in a previous study involving milk (Holtzapple et al., 1999), matrix components that are retained on the IAC column and elute to the RP column adversely affect the chromatographic profile. Although the performance of the RP column can be restored, it is advantageous to minimize the amount of contaminating matrix components that are retained on the IAC column, thus decreasing the amount of time necessary to restore the RP column. A number of additions to the samples or to the binding buffer were investigated for their ability to prevent binding of matrix components to the IAC column. These include addition of anti-aflatoxin antibody A1 (Holtzapple et al., 1996), 0.1%

Table 1. Linear Regression Data and Square of Correlation Coefficients for the Fluorescence Standard Curves of Four Fluoroquinolones

	line equation: response (y) = m(x ng/mL) + b		
fluoroquinolone	m	b	r^2
CIPRO	4770	17	0.9999
ENRO	7827	−7.4	0.9996
SARA	3024	32	0.9967
DI	5207	−476	0.9996

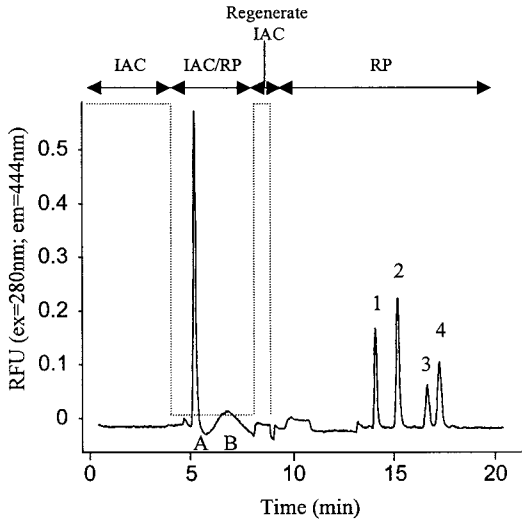

Figure 3. Representative IAC/RP/fluorescence chromatogram showing the column switching events (lines with double arrows) and the elution profile (with the dotted line indicating the percent of binding buffer): (1) ciprofloxacin; (2) enrofloxacin; (3) sarafloxacin; (4) difloxacin.

Tween 20, and up to 10% methanol. Because it was possible that the matrix components were nonspecifically binding to the antibodies on the column, the addition of soluble antibodies to the samples may have lessened or prevented binding of the matrix to the bound anti-sarafloxacin antibodies. However, inclusion of these antibodies in the samples did not decrease matrix binding. Effective extraction of the fluoroquinolones and removal of most of the nonspecific binding were finally achieved by the addition of 5% methanol (final concentration) to the samples.

Calibration curves were determined for ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin using 100-μL injections of mixtures containing 1, 2, 5, or 10 ng/mL of each of the fluoroquinolones. Linear regression data for typical standard curves obtained for the fluoroquinolones are listed in Table 1. Figure 3 shows a representative IAC/RP/fluorescence chromatogram of the fluoroquinolones at 5 ng/mL that relates the chromatographic events to the column switching and elution events. Peaks A and B represent the fluoroquinolones as they elute from the IAC column. As can be clearly seen, the four fluoroquinolones elute in two distinct peaks, with enrofloxacin and ciprofloxacin eluting in peak A and sarafloxacin and difloxacin eluting in peak B. The separation of these drugs on the IAC column is due to differences in their relative affinities for Sara-95. Enrofloxacin and ciprofloxacin (ELISA IC_{50} values ~ 125−150 ng/mL) exhibit a ~15-fold lower relative affinity for Sara-95 than sarafloxacin and difloxacin (ELISA IC_{50} values ~ 5−10 ng/mL) and, therefore, are the first to elute from the IAC column.

Figure 4 shows the chromatograms obtained for a liver blank (A) and a liver extract fortified with 50 ng/g

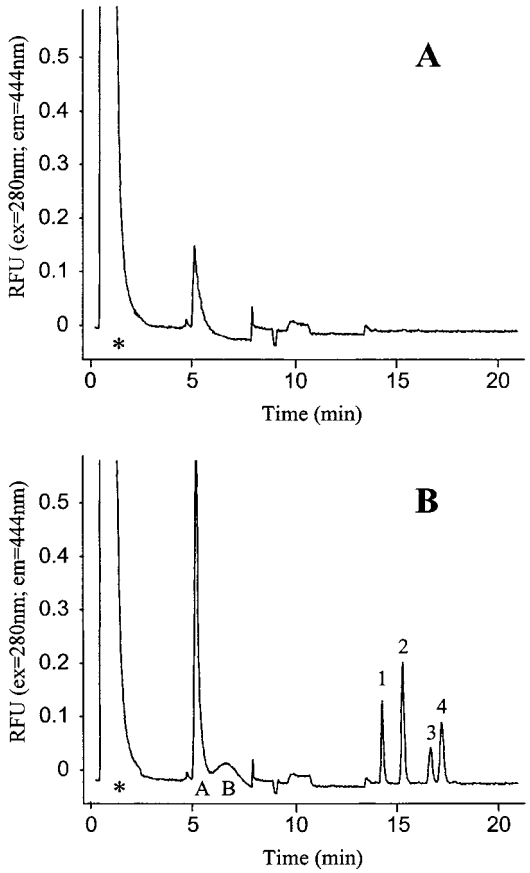

Figure 4. Chromatograms of (A) a blank liver extract and (B) a liver extract from a sample fortified with 50 ng/g (5 ng/mL final concentration in the extract) of each fluoroquinolone: (1) ciprofloxacin; (2) enrofloxacin; (3) sarafloxacin; (4) difloxacin.

of each fluoroquinolone (B). As can be observed, most of the liver matrix components elute immediately after injection of the samples onto the IAC column (Figure 4A, peak designated with an asterisk). The response essentially returns to background after the IAC column is washed with 40 CV of PBS and 10 CV of 0.1 M sodium phosphate buffer (pH 6.0). Upon application of the elution buffer to the column, it is evident that a small amount of liver matrix components nonspecifically adsorbs to the IAC column (Figure 4A, peak A eluting at ~5 min) despite the addition of methanol to the extraction buffer. Although these components elute at the same time from the IAC column as the less strongly held enrofloxacin and ciprofloxacin, they do not prevent analysis of the four fluoroquinolones on the RP column as shown in Figure 4B. The peaks corresponding to (1) ciprofloxacin, (2) enrofloxacin, (3) sarafloxacin, and (4) difloxacin exhibited retention times of 14.1, 15.1, 16.5, and 17.0 min, respectively. The liver components did not elute from the RP column under the conditions used, even after automated repeat injections of liver samples (at least 40).

Even though these components did not interfere with quantification of the fluoroquinolones, they were responsible for the increase in the RP column back pressure that was observed during the injection of ~40 samples. Simple replacement of in-line filters brought the pressure back down to that observed at the beginning of the study. To maintain column performance, the RP column was washed periodically according to the manufacturer's instructions to prevent buildup of phos-

Table 2. Average Recovery of Fluoroquinolones from Fortified Liver Samples

	recovery, %, at indicated fortification level[a]								
	20 ng/g			50 ng/g			100 ng/g		
	mean	SD	CV	mean	SD	CV	mean	SD	CV
CIPRO	90.6	1.0	1.1	88.2	0.6	0.7	85.9	2.7	3.2
ENRO	92.6	2.8	3.0	93.5	3.2	3.0	90.7	4.3	4.8
SARA	89.1	0.8	0.9	89.3	1.0	1.1	85.7	1.9	2.3

[a] Each mean is the average of three separate experiments. For each experiment, triplicate samples were analyzed for each fluoroquinolone at each fortification level.

phate salts or matrix components. No special treatment of the IAC column was required to maintain column performance other than storage at 4 °C in PBS containing 0.02% sodium azide. The reusability and durability of the IAC column are important aspects of this method in that consistent results can be obtained for multiple samples using a single column. IAC columns in our laboratory have retained consistent analyte binding capabilities even after being used for hundreds of samples over a period of up to 1 year.

The percent recovery of the four fluoroquinolones from fortified liver is reported in Table 2. Samples were fortified separately in triplicate with each of the fluoroquinolones and assayed on three separate days. Recovery calculations were based upon fluoroquinolone standard curves that were generated for each day's experiment. A mean intraassay coefficient of variation (CV) of 3.2% (12 observations per day; $n = 12$) and a mean interassay CV of 2.1% (3 days with 12 observations per day; $n = 36$) were observed. The variability at each fortification level is demonstrated by CV values presented in Table 2. The limit of quantification (LOQ), which is limited by the lowest concentration point used to generate the standard curve for each of the fluoroquinolones, is 1 ng/mL. The limits of detection (LOD), based on a signal-to-noise ratio of 5:1, were 0.47, 0.32, 0.87, and 0.53 ng/mL (47, 32, 87, and 53 pg per 100-μL injection) for ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin, respectively. Because the liver extraction procedure involves a 10-fold dilution, the corresponding LOD values in liver are 4.7, 3.2, 8.7, and 5.3 ng/g (470, 320, 870, and 530 pg/100-μL injection).

Although the method exhibits good sensitivity for all four fluoroquinolones, greater sensitivity can be achieved if necessary by simply increasing the volume of the sample loop that is used for injections. Due to the ability of the IAC column to concentrate fluoroquinolones prior to elution, volumes from 10 to almost 2000 μL (the capacity of the autosampler vial) can be injected. If even greater sensitivity is required, multiple injections of the same sample (using multiple autosampler vials) can be applied to the IAC column prior to elution of the captured fluoroquinolones to the RP column. The method, therefore, has a broad dynamic range because it is not limited with regard to sample size.

CONCLUSIONS

The two-column automated method presented here is capable of detecting fluoroquinolones in chicken liver at low nanograms per gram levels. In addition, use of an on-line, fluoroquinolone-specific immunoaffinity column reduced both the number of sample preparation steps and the amount of organic solvent required for analyses. Incorporation of this method into a residue-monitoring program has the potential to decrease the amount of time and labor involved in screening liver samples for these antibiotics.

The ability of an IAC column to separate compounds due to differences in their relative affinities for the antibody may provide the basis for detection methods that require only an immunoaffinity column for sample analyses. Detection methods based on this type of format would not require organic solvents for extraction or separation of analytes, thus minimizing the amount of organic waste and reducing the costs associated with analyses. Further studies investigating the development of detection methods such as these are ongoing in our laboratory.

ACKNOWLEDGMENT

We thank D. Peterson for helpful discussions concerning statistical analysis of the data.

LITERATURE CITED

Gau, W.; Plaschke, H. J.; Schmidt, K.; Weber, B. Determination of ciprofloxacin (Bay o 9867) in biological fluids by high performance liquid chromatography. *J. Liq. Chromatogr.* **1985**, *8*, 485−498.

Holtzapple, C. K.; Stanker, L. H. Affinity selection of compounds in a fluoroquinolone chemical library by on-line immunoaffinity deletion coupled to column HPLC. *Anal. Chem.* **1998**, *70*, 4817−4821.

Holtzapple, C. K.; Carlin, R. J.; Rose, B. G.; Kubena, L. F.; Stanker, L. H. Characterization of monoclonal antibodies to aflatoxin M1 and molecular modeling studies of related aflatoxins. *Mol. Immunol.* **1996**, *33*, 939−946.

Holtzapple, C. K.; Buckley, S. A.; Stanker, L. H. Production and characterization of monoclonal antibodies against sarafloxacin and cross-reactivity studies of related fluoroquinolones. *J. Agric. Food Chem.* **1997**, *45*, 1984−1990.

Holtzapple, C. K.; Buckley, S. A.; Stanker, L. H. Determination of four fluoroquinolones in milk by on-line immunoaffinity capture coupled with reversed-phase liquid chromatography. *J. AOAC Int.* **1999**, in press.

Horie, M.; Saito, K.; Nose, N.; Nakazawa, H. Simultaneous determination of benofloxacin, danofloxacin, enrofloxacin and ofloxacin in chicken tissue by high-performance liquid chromatography. *J. Chromatogr., B: Biomed. Appl.* **1994**, *653*, 69−76.

Hormazabal, V.; Yndestad, M. Rapid assay for monitoring residues of enrofloxacin in milk and meat tissues by HPLC. *J. Liq. Chromatogr.* **1994**, *17*, 3775−3782.

Kaartinen, L.; Salonen, M.; Älli, L.; Pyörälä, S. Pharmacokinetics of enrofloxacin after single intravenous, intramuscular and subcutaneous injections in lactating cows. *J. Vet. Pharmacol. Ther.* **1995**, *18*, 357−362.

Lynch, M. J.; Rice, J. R.; Ericson, J. F.; Mosher, F. R.; Millas, W. J.; Harran, L. P.; Frame, G. M.; Illyes, E. F.; McGuirk, P. R.; Jefson, M. R.; Risk, J. E.; Magonigle, R. A. Residue depletion studies on danofloxacin in the chicken. *J. Agric. Food Chem.* **1984**, *42*, 289−294.

Morton, S. J.; Shull, V. H.; Dick, J. D. Determination of norfloxacin and ciprofloxacin concentrations in serum and urine by high-pressure liquid chromatography. *Antimicrob. Agents Chemother.* **1986**, *30*, 325−327.

Nedved, M. L.; Habibi-Goudarzi, S.; Ganem, B.; Henion, J. D. Characterization of benzodiazepine "combinatorial" chemical libraries by on-line immunoaffinity extraction, coupled column HPLC-ion spray mass spectrometry-tandem mass spectrometry. *Anal. Chem.* **1996**, *68*, 4228−4236.

Newkirk, D. K.; Benson, R. W.; Howard, P. C.; Churchwell, M. I.; Doerge, D. R.; Roberts, D. W. On-line immunoaffinity capture, coupled with HPLC and electrospray ionization

mass spectrometry, for automated determination of fumonisins. *J. Agric. Food Chem.* **1998**, *46*, 1677−1688.

Roybal, J. E.; Pfenning, A. P.; Turnipseed, S. B.; Walker, C. C.; Hurlbut, J. A. Determination of four fluoroquinolones in milk by liquid chromatography. *J. AOAC Int.* **1997**, *80*, 982−987.

Studer-Rohr, I.; Dietrich, D. R.; Schlatter, J.; Schlatter, C. The occurrence of ochratoxin A in coffee. *Food Chem. Toxicol.* **1995**, *33*, 341−355.

Tarbin, J. A.; Tyler, D. J.; Shearer, G. Analysis of enrofloxacin and its metabolite ciprofloxacin in bovine and porcine muscle by high-performance liquid chromatography following cation exchange clean up. *Food Addit. Contam.* **1992**, *9*, 345−350.

Tuinstra, L. G. M. Th.; Roos, A. H.; van Trijp, J. M. P. Liquid chromatographic determination of aflatoxin M1 in milk powder using immunoaffinity columns for cleanup: Interlaboratory study. *J. AOAC Int.* **1993**, *76*, 1248−1254.

Tyczkowska, K. L.; Voyksner, R. D.; Anderson, K. L.; Papich, M. G. Simultaneous determination of enrofloxacin and its primary metabolite ciprofloxacin in bovine milk and plasma by ion-pairing liquid chromatography. *J. Chromatogr., B: Biomed. Appl.* **1994**, *658*, 341−348.

Vanderlaan, M.; Hwang, M.; Djanegara, T. Immunoaffinity purification of dietary heterocyclic amine carcinogens. In *Biomarkers in Human Cancer? Part II. Exposure Monitoring and Molecular Dosimetry*; Bartsch, H., Kadlubar, F., O'Neill, I., Eds.; National Institutes of Health: Bethesda, MD, 1993; pp 285−287.

Waggoner, T. B.; Bowman, M. C. Spectrofluorometric determination of BAY Vp 2674 residues in poultry tissues. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 813−818.

Received for review February 22, 1999. Revised manuscript received April 28, 1999. Accepted May 2, 1999. Mention of a tradename, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

JF990200Y