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Determination of fluoroquinolones in serum using an on-line clean-up column coupled to high-performance immunoaffinity–reversed-phase liquid chromatography

Carol K. Holtzapple*[,1], Sandra A. Buckley, Larry H. Stanker[2]

Food Animal Protection Research Laboratory, Agricultural Research Service, US Department of Agriculture, 2881 F&B Road, College Station, TX 77845, USA

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Abstract

A simple, rapid and reliable method for the simultaneous analysis of the fluoroquinolones ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin in bovine serum has been developed. Upon injection of serum samples, an on-line protein G-linked column was employed to automatically remove serum components that otherwise would interfere with analyses. A high-performance immunoaffinity chromatography (HPIAC) column containing covalently bound anti-sarafloxacin antibodies was then used to capture the fluoroquinolones while allowing the remainder of the serum components to elute to waste. After binding to the HPIAC column, the fluoroquinolones were eluted directly onto a reversed-phase (RP) column for final separation of the compounds prior to fluorescence detection at excitation and emission wavelengths of 280 and 444 nm, respectively. Due to use of a clean-up column in tandem with a highly selective HPIAC column, the only off-line sample preparation required was dilution (10-fold) in phosphate buffered saline (PBS) and passage of the samples through a 0.2-μm filter to remove particulate matter prior to injection. No significant interferences from the sample matrix were observed, indicating good selectivity with the HPIAC column. The method yielded high recoveries from fortified bovine serum that were >95% for all four fluoroquinolones with good reproducibility (C.V. values <7.0%). The on-line, automated method described here provides a simple, sensitive and specific assay for multiresidue detection of fluoroquinolones in serum.

Keywords: Fluoroquinolones

1. Introduction

The fluoroquinolones are a group of structurally related synthetic antibacterial agents. They are derived from the quinolone nalidixic acid, and exert their therapeutic effects by inhibiting DNA gyrase within the bacterial cell [1]. The carboxylic acid at position 3 (see Fig. 1 for fluoroquinolone structures) and the ketone group at position 4 are necessary for DNA gyrase inhibition, whereas substitutions at positions 1 and 7 influence the potency and biological spectrum of activity of the drugs. Sarafloxacin was the first of these compounds to be approved

*Corresponding author. Fax: +1-409-260-9332.

E-mail address: ffsru@ffsru.tamu.edu (C.K. Holtzapple).

[1]Present address: ScienceWorks, 1805 Southwood Drive, College Station, TX 77840, USA.

[2]Present address: Western Regional Research Center, Agricultural Research Service, US Department of Agriculture, 800 Buchanan Street, Albany, CA 94710, USA.

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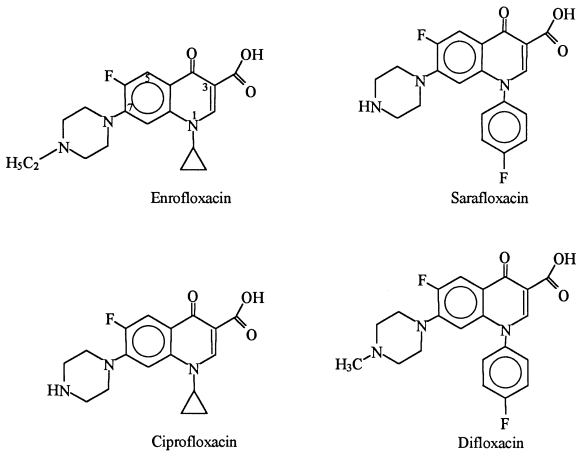

Fig. 1. Structures of the fluoroquinolones evaluated in this study.

for use in food animals. Subsequently, enrofloxacin was approved for use in cattle, chickens and turkeys with regulatory residue tolerance levels set at 100 (liver), 300 (muscle) and 300 (muscle) ng/ml, respectively. Prompted by concerns that the effectiveness of this valuable class of antibiotics could be reduced if resistant pathogens developed in the treated animal population and were transferred to the human population, regulatory agencies as well as the manufacturer of the drug implemented a post-approval monitoring program. The objective of the program is to determine whether use of this class of drugs in food and/or non-food animals presents a risk to public health. To meet this objective, careful surveillance of the development of resistant organisms and of the presence of fluoroquinolone residues in animals or animal products is required. In order to carry out an effective residue detection program involving thousands of samples, a rapid detection method for the fluoroquinolones must be available.

A number of analytical high-performance liquid chromatography (HPLC) methods have been published which describe the analysis of quinolones [2–6]. Because these methods are labor intensive, requiring extensive sample clean-up procedures, they cannot be used for routine screening of numerous samples. We recently developed a monoclonal antibody-based, enzyme-linked immunosorbent assay (ELISA) capable of detecting fluoroquinolones in chicken liver [7]. This assay circumvented the problem of laborious sample clean-up procedures and provided a simple method to screen numerous samples for the presence of fluoroquinolone residues. Immunoassays are particularly suitable for use in routine surveillance programs due to the simplicity, sensitivity and selectivity of the assays. Although useful for rapid screens, immunoassays in general have not been used as analytical methods because most antibodies, even those of monoclonal origin, exhibit broad cross-reactivity against structurally similar compounds within a class of compounds. The consequence of cross-reactivity is that it is not obvious whether the result obtained by immunoassay is due to a small amount of high-affinity analytes, a mixture of high- and low-affinity compounds, or a large amount of low-affinity cross-reacting substances [8]. As a result, samples that test positive in an immunoassay (initial screen) must undergo further analysis using conventional HPLC methods that require extensive sample clean-up procedures.

The ideal method of analysis would be one that takes advantage of the strengths of both immunochemistry- and liquid chromatography-based analytical methods. Such a method would require minimal sample preparation, and would be capable of identifying all of the compounds of interest. Methods incorporating off-line immunoaffinity columns have been used to prepare samples prior to injection into an HPLC system, thus providing cleaner extracts and minimizing the level of interference introduced into the system by co-extracted compounds present in complex matrices [9].

More recently, methods incorporating high-performance immunoaffinity columns (HPIAC) linked on-line with HPLC columns have been developed that take advantage of the minimal sample preparation required by immunoassays and the compound separation capabilities of HPLC analyses. Such methods have been used to capture and quantify compounds from a variety of sources including combinatorial libraries [10], rodent feed [8], equine urine [11], bovine milk [12] and chicken liver [13]. Although the sample clean-up procedures for these methods are not as laborious as those required for traditional HPLC methods, samples of the more complex matrices (feed, milk and liver) needed more preparation than that required for immunoassay analyses. Either off-line sample clean-up procedures (rodent feed) or frequent reversed-phase column regeneration (milk and liver) were required to remove contaminating matrix components.

In this paper, we describe a method that incorporates an on-line clean-up column in tandem with HPIAC–reversed-phase (RP) chromatography to overcome the difficulties encountered with analyzing complex matrices. The clean-up column traps serum components that otherwise would bind to the HPIAC column and co-elute with the fluoroquinolones to the RP column; thus, the clean-up column reduces fouling of the RP column. The HPIAC column effectively captures the fluoroquinolones, while the remainder of the serum matrix elutes to waste. The fluoroquinolones then elute from the HPIAC to the RP column for final separation prior to fluorescence detection. This automated, on-line, multiresidue detection method for the fluoroquinolones is simple to perform, applicable to routine analysis, and capable of resolving the fluoroquinolones in serum without interference from serum matrix components.

2. Experimental

2.1. Analytical reference standards: fluoroquinolones

Sarafloxacin (885 μg/mg) and difloxacin (902 μg/mg) were gifts from Abbott Laboratories (North Chicago, IL). Enrofloxacin (999 μg/mg) and ciprofloxacin (848 μg/mg) were gifts from Bayer (Kansas City, MO).

2.2. Solvents and reagents

For liquid chromatographic analyses, HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ). Glacial acetic acid and monobasic sodium phosphate were purchased from Sigma (St. Louis, MO). BupH™ phosphate buffered saline packs were purchased from Pierce (Rockford, IL). For cross-linking the antibody to the immunoaffinity column, dimethyl pimelimidate, monoethanolamine, and triethanolamine were purchased from Sigma.

2.3. HPLC columns

The clean-up and HPIAC columns were made by packing PEEK cartridges (2.1×30 mm D×L) with POROS™ media (PE Biosystems; Framingham, MA) containing protein G covalently bound to its surface. No further treatment of the clean-up column was required. To make the HPIAC column, monoclonal antibody Sara-95 (Mab Sara-95; [7]) was purified by low-pressure protein G column chromatography, dialyzed against PBS (150 m*M* NaCl, 100 m*M* phosphate, pH 7.2), and brought to a final concentration of 2 mg/ml in PBS. The antibody was adsorbed to the protein G on the surface of the POROS™ XL media, and then covalently cross-linked in place according to the manufacturer's instructions. Both columns were stored in PBS/0.02% sodium azide to prevent microbial contamination. An Inertsil phenyl column (5 μm, 150×4.6 mm; Alltech, Deerfield, IL) was used for final separation of the fluoroquinolones prior to fluorescence detection.

2.4. HPLC system

The chromatographic system used is an Integral Microanalytical Workstation from PE Biosystems (Framingham, MA). The system is equipped with an autosampler, two HPLC pumps, three 10-port switching valves, two reagent syringe pumps, a fluorescence detector and a variable wavelength UV detector. The three columns (clean-up, HPIAC, and RP) were linked as shown in Fig. 2. Pump 1 delivered the binding buffers (PBS or 0.1 *M* NaH_2PO_4, pH 6.0) for the HPIAC column. Pump 2 delivered the elution buffer (2% [v/v] acetic acid, acidified with HCl to obtain pH 2.2) for the HPIAC column and the mobile phase (elution buffer/acetonitrile, 85:15 [v/v]) for the RP column. Selective capture of the fluoroquinolones on the HPIAC column was performed at room temperature, and separation on the RP column was accomplished at 40°C using a Golden Foil™ column heater (Alltech; Deerfield, IL). For fluorescence detection, the excitation and emission wavelengths were set at 280 and 444 nm, respectively.

Using the column switching capabilities of the Integral system, the fluoroquinolones were automatically captured on the HPIAC column, eluted from the HPIAC column to the RP column, and finally separated on the RP column prior to fluorescence

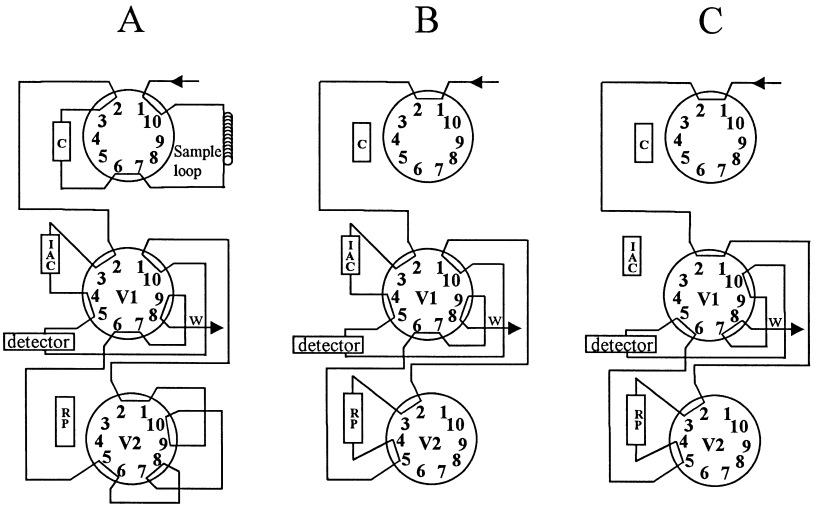

Fig. 2. Plumbing diagrams for the Integral Microanalytical Workstation. Direction of flow for (A) loading the samples or standards and for reconditioning the clean-up and HPIAC columns, (B) eluting the fluoroquinolones from the HPIAC column to the second column, (C) separating the fluoroquinolones on the RP column.

detection. The plumbing diagram for the system is shown in Fig. 2. In the first step (Fig. 2A), both the clean-up and the HPIAC columns were placed in-line and equilibrated with PBS at 3 ml/min. The fluoroquinolones were then injected using the autosampler and a 100-μl sample loop. Both columns were washed with 10-column volumes (1 ml) of PBS at 0.5 ml/min, 10-column volumes of PBS at 2 ml/min and then 10-column volumes of 0.1 *M* NaH_2PO_4 (pH 6) at 2 ml/min. Serum components that would have bound nonspecifically to sites on the HPIAC column were retained on the clean-up column whereas the fluoroquinolones passed through the clean-up column and bound specifically to Mab Sara-95 that was covalently bound to the HPIAC column. Serum components that did not bind to either column were flushed to waste.

In the second step (Fig. 2B), the HPIAC and RP columns were placed in-line and elution of the fluoroquinolones from the HPIAC column to the RP column was accomplished using an isocratic elution step with elution buffer at 0.5 ml/min for 2 min. The RP column was then placed off-line.

In the third step (Fig. 2A), the clean-up and HPIAC columns were switched in-line and the contaminating serum components on the clean-up column were flushed to waste using 2 ml elution buffer at 3 ml/min. Both columns were then reequilibrated with 3 ml PBS at 3 ml/min.

Finally (Fig. 2C), the RP column alone was placed in-line and separation of the fluoroquinolones was accomplished isocratically using the RP mobile phase with fluorescence detection.

2.5. Standard curve solutions

Stock fluoroquinolone solutions were prepared by dissolving each fluoroquinolone in methanol at 1 mg/ml. Intermediate standards were prepared by diluting stock solutions in PBS to obtain concentrations of 1, 5, 10 and 100 μg/ml. The intermediate standards were then used to make analytical standards at concentrations between 1 and 10 000 ng/ml.

2.6. Fortified serum

The concentrations of fluoroquinolones in serum can range between 1100 (30 min post-treatment) and 180 ng/ml (12 h post-treatment). Therefore, samples (1 ml) of fluoroquinolone-free bovine serum were spiked with intermediate standards of sarafloxacin, difloxacin, enrofloxacin, and ciprofloxacin to obtain concentrations of 20, 50 or 100 ng/ml. These concentrations represent 1/5×-, 1/2×-, and 1×-

tolerance levels specified for enrofloxacin analyses, respectively. Because the tolerance level is based on a metabolite of enrofloxacin, desethylciprofloxacin, for regulatory purposes, screening methods must be able to detect the parent compound at 1/4× tolerance level (C. Deyrup, Food Safety and Inspection Service, personal communication). The spiked samples were diluted 10-fold in PBS and filtered through a 0.2-μm filter directly into autosampler vials for HPLC analysis.

2.7. Percent recovery

Percent recovery values were determined by comparing the observed concentrations in fortified serum with the calibration standards in PBS.

2.8. Incurred enrofloxacin residues in serum

One angus-cross bull calf was given a single dose of enrofloxacin (5 mg/kg) according to the manufacturer's instructions. Serum samples were obtained at 1, 4, 8, 12, and 24 h post-treatment. A final sample was obtained at 1 week post-treatment. These samples were prepared for HPLC analysis as previously described for the spiked samples.

3. Results and discussion

3.1. HPIAC–RP method characteristics

The HPIAC–RP method of detecting fluoroquinolones was evaluated for ciprofloxacin, enrofloxacin, sarafloxacin and difloxacin in bovine serum samples. The time needed for each sample preparation was less than 1 min due to the on-line clean-up and HPIAC columns. Therefore, the number of samples that can be processed in 24 h is not limited by the time required for sample preparation but by the 20-min analysis time required by the method. Approximately 72 samples can be analyzed in a 24-h period. The rigid, porous support used for the HPIAC column allowed the use of slow flow-rates for fluoroquinolone binding and fast flow-rates for column washing/reequilibration, thus minimizing the time required for each analysis. All four fluoroquinolones exhibited good binding to Mab Sara-95 even after washing with 20-column volumes of PBS and 0.1 *M* NaH_2PO_4 (pH 6) at 2.0 ml/min.

An important aspect of the method is the reusability/durability of the HPIAC column that allows generation of consistent results for multiple samples using a single column. No special treatment of the column was required to maintain column performance other than storage at 4°C in PBS containing 0.02% sodium azide. The column was operated at room temperature and retained consistent analyte binding capabilities even after being used for hundreds of samples over a period of 6 months.

3.2. Calibration curves

The calibration curves were linear for the calibration range of 1–1000 ng/ml used to calculate the percent recoveries from serum. A representative chromatogram obtained with the calibration standard containing 10 ng/ml of each fluoroquinolone is shown in Fig. 3. Antigen saturation studies to investigate the maximum binding capacity of the immunoaffinity column demonstrated that the column could bind a total of 6500 ng of fluoroquinolones before its capacity was exceeded. If all four fluoroquinolones were present in a standard at high levels (10 000 ng/ml), the two fluoroquinolones (sarafloxacin and difloxacin) exhibiting the highest

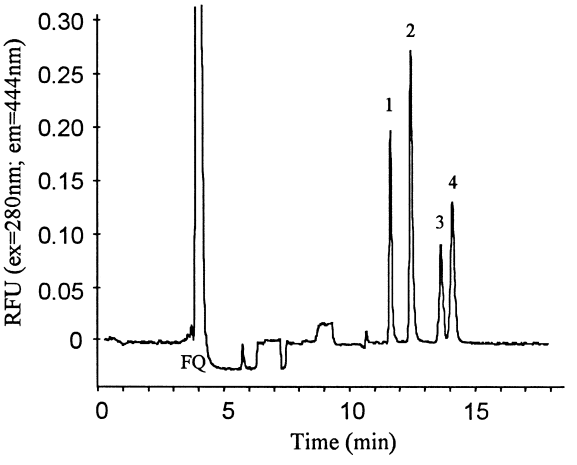

Fig. 3. Representative HPIAC–RP–fluorescence chromatogram obtained with the calibration standard containing 10 ng/ml of each fluoroquinolone. 1, ciprofloxacin; 2, enrofloxacin; 3, sarafloxacin; 4, difloxacin.

relative affinities for the antibody preferentially bound to the HPIAC column. The relative affinities of sarafloxacin and difloxacin for Mab Sara-95 are approximately 15-fold greater than those of ciprofloxacin and enrofloxacin as determined previously by competitive binding studies using an ELISA format [7]. Representative chromatograms in Fig. 4 demonstrate the different chromatographic profiles obtained when standards containing 1000 or 10 000 ng/ml of each fluoroquinolone were analyzed by HPIAC–RP–fluorescence. The peak marked with an asterisk (*) represents fluoroquinolones that were unable to bind because the amount injected exceeded the capacity of the column.

3.3. Analysis of spiked bovine serum

Bovine serum samples spiked with four fluoroquinolones were analyzed using the on-line HPIAC–RP–fluorescence method described herein. Fig. 5 shows the chromatograms obtained for a serum blank (A) and a serum sample fortified with 50 ng/ml of

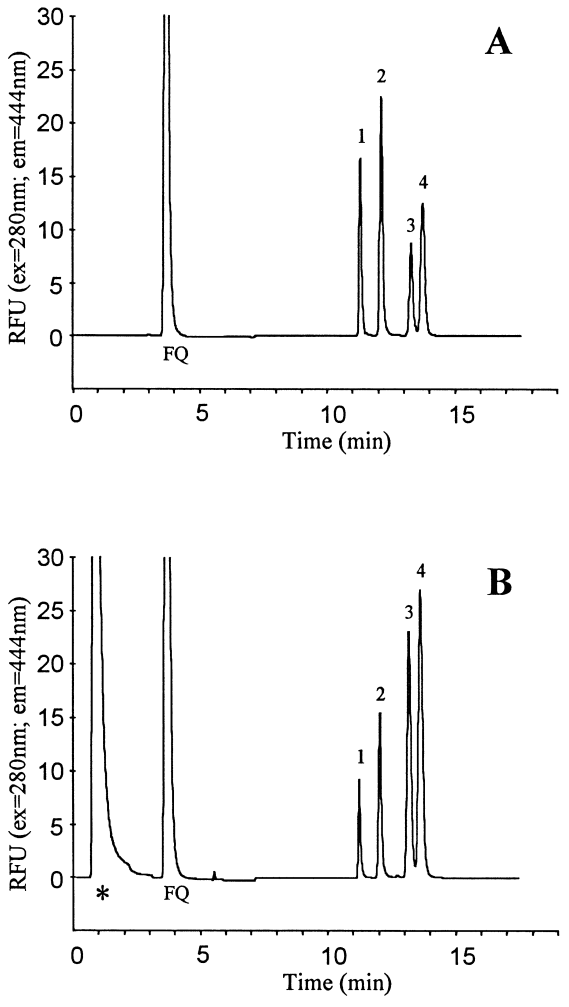

Fig. 4. Representative chromatograms obtained with calibration standards containing (A) 1000 ng/ml or (B) 10 000 ng/ml of each fluoroquinolone. 1, ciprofloxacin; 2, enrofloxacin; 3, sarafloxacin; 4, difloxacin.

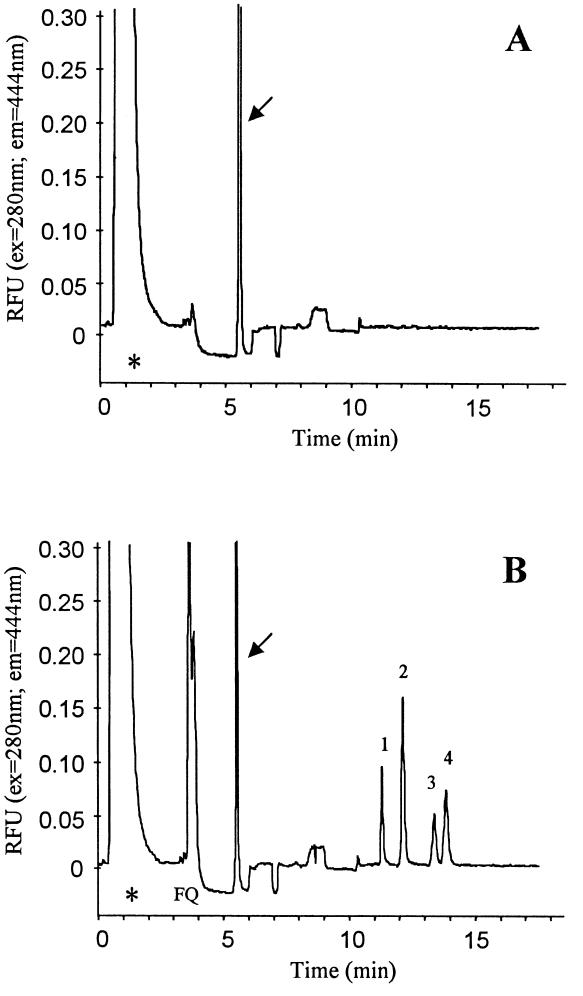

Fig. 5. Representative chromatograms of (A) a blank serum sample and (B) a serum sample fortified with 50 ng/ml of each fluoroquinolone. Due to the 10-fold serum dilution, the final injected concentration was 5 ng/ml for each fluoroquinolone. 1, ciprofloxacin; 2, enrofloxacin; 3, sarafloxacin; 4, difloxacin.

each fluoroquinolone (B). As can be observed, most of the serum matrix components elute immediately after injection of the samples through the clean-up and HPIAC columns (Fig. 5A and B, peaks designated with asterisks). After washing both columns with 20-column volumes of PBS and 10-column volumes of 0.1 *M* NaH_2PO_4 (pH 6.0), the response essentially returns to background levels. The clean-up column was taken off-line and elution buffer was applied to elute the bound fluoroquinolones from the HPIAC column to the RP column. The two closely associated peaks marked with ''FQ'' in Fig. 5B represent the four fluoroquinolones eluting from the HPIAC column. The RP column was then placed off-line so that the sample components adhering to the clean-up column could be removed. The peaks marked with arrows (Fig. 5A and B) represent elution of these components from the clean-up column to waste. Removal of these interfering matrix components prevented the undesirable increase in the RP column back pressure that was observed when a clean-up column was not used, thus maintaining RP column performance without the need for numerous column washes.

The percent recovery values for the four fluoroquinolones at three different fortification levels in bovine serum are presented in Table 1. Recovery calculations were based upon fluoroquinolone standard curves that were generated for each day's experiment. The average recovery values for ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin were 99.2, 102.3, 97.8, and 101.4%, respectively. A mean intraassay coefficient of variation (C.V.) of 3.0% (12 observations per day; $n=12$) and a mean inter-assay C.V. of 4.9% (3 days with 12 observations per day; $n=36$) were observed. The variability at each fortification level is demonstrated by C.V. values presented in Table 1.

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. Antigen dilution studies were used to determine the lower limit of detection (LOD). Based on a signal-to-noise ratio of 5:1, the LOD values were 0.32, 0.18, 0.47, and 0.28 ng/ml (32, 18, 47, and 28 pg per 100-μl injection) for ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin, respectively. Because the serum is diluted 10-fold prior to injection, the corresponding LOD values in serum are 3.2, 1.8, 4.7, and 2.8 ng/ml (320, 180, 470, and 280 pg per 100-μl injection). Greater sensitivity for fluoroquinolones in serum can be achieved by simply increasing the volume of the sample loop or by applying multiple injections of the same sample prior to elution of the captured fluoroquinolones from the HPIAC column to the RP column. Therefore, due to the ability of the HPIAC column to concentrate analytes prior to elution, the method has a broad dynamic range that is not limited with regard to sample size.

3.4. Analysis of incurred enrofloxacin residues in bovine serum

The applicability of this method for detecting residues in the serum of fluoroquinolone-treated animals was demonstrated using enrofloxacin. Samples containing the highest, lowest, and undetectable

Table 1
Average recovery of fluoroquinolones from fortified serum samples

FQ	Recovery, percentage at indicated fortification level[a]								
	20 ng/g			50 ng/g			100 ng/g		
	Mean	SD	C.V.	Mean	SD	C.V.	Mean	SD	C.V.
CIPRO	100.3	2.6	2.6	97.0	0.3	0.4	100.3	3.5	3.4
ENRO	100.6	2.4	2.4	105.2	4.7	4.4	101.3	2.3	2.3
SARA	95.0	5.5	5.8	100.6	5.0	5.0	97.9	1.0	1.0
DI	100.8	6.6	6.5	102.6	5.5	5.3	100.7	1.9	1.9

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

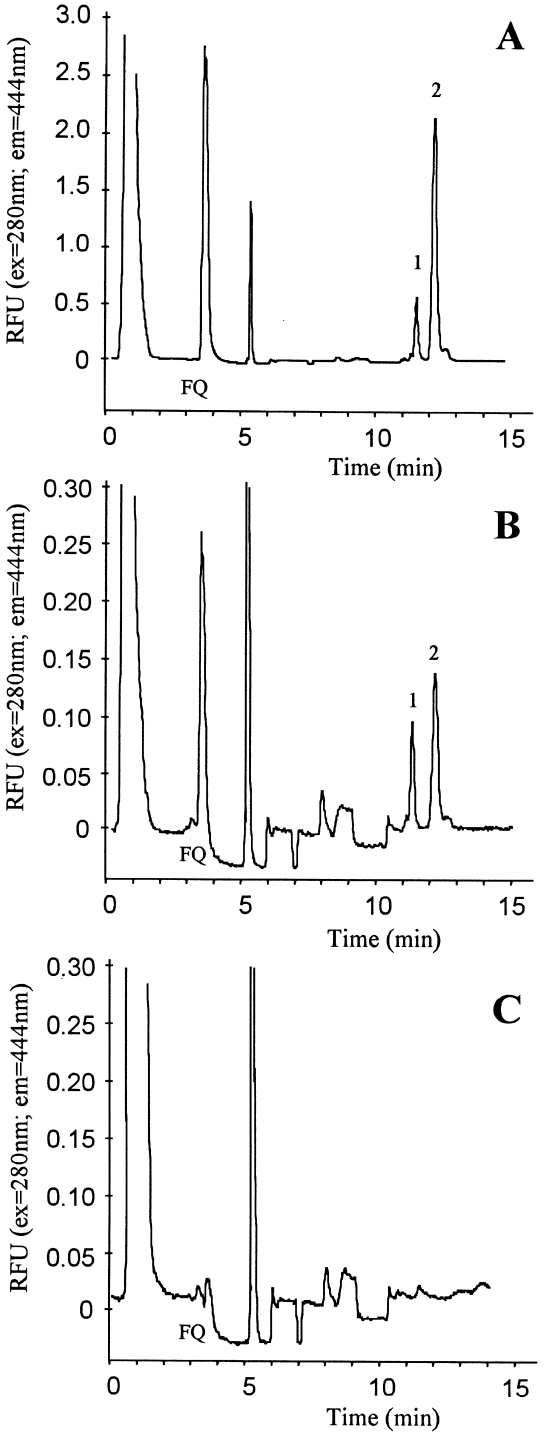

Fig. 6. Representative chromatograms obtained by analysis serum samples containing incurred enrofloxacin residues. Samples were collected at (A) 4 h, (B) 24 h, and (C) 1 week post-treatment. 1, ciprofloxacin; 2, enrofloxacin.

levels of enrofloxacin were analyzed. Fig. 6 shows the HPIAC–RP–fluorescence chromatograms resulting from analysis of these serum samples collected at (A) 4 h (796 ng/ml enrofloxacin), (B) 24 h (52 ng/ml enrofloxacin) and (C) 1 week (below the limit of detection) post-treatment. The chromatograms demonstrate that the peaks for enrofloxacin and its metabolite ciprofloxacin do not interfere with each other and that the method is sufficiently sensitive to detect concentrations of these fluoroquinolones likely to be encountered in the field.

4. Conclusions

This method demonstrates simultaneous determination of four fluoroquinolones in fortified bovine serum using an automated column switching system for on-line sample clean-up and HPIAC extraction coupled to RP column chromatography. All four fluoroquinolones could be detected and quantified at low nanograms per milliliter (ppb) levels. Use of an on-line clean-up column in tandem with a fluoroquinolone-specific immunoaffinity column reduced the number of sample preparation steps and the amount of organic solvent required for each analysis. Thus, this method takes advantage of the strengths of both immunochemistry- and LC-based separation strategies and has the potential to decrease the amount of time and labor involved in screening serum samples for these antibiotics.

Acknowledgements

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