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Simultaneous determination of danofloxacin and N-desmethyldanofloxacin in cattle and chicken edible tissues by liquid chromatography with fluorescence detection

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Abstract

A rugged, simple, and selective method for the determination of danofloxacin and its primary metabolite, N-desmethyldanofloxacin, in cattle (liver, muscle, kidney, and fat) and chicken (liver and muscle) tissues was developed. The method is selective for danofloxacin and N-desmethyldanofloxacin over other veterinary important fluoroquinolones, such as enrofloxacin, ciprofloxacin, norfloxacin, and ofloxacin. Selectivity is achieved through a combination of extraction, chromatography, and fluorescence detection. The analytes were extracted from homogenized tissues using a methanol–perchloric–phosphoric acid solution. After centrifugation, direct injection of extraction supernate was possible. The limit of quantitation was 20 pg on column. Separation was achieved on an Inertsil C_8 (5 μm, 100 Å) column with dimensions of 250×4.6 mm I.D. The mobile phase consisted of 0.05 *M* phosphate buffer (pH 3.5)–acetonitrile (88:12). A fluorescence detector was utilized with an excitation wavelength of 280 nm and an emission wavelength of 440 nm. The assay was accurate and reproducible within the range of 10 to 500 ng/g for both danofloxacin and N-desmethyldanofloxacin. Intra-assay accuracy was between 98 and 101%, and precision was less than 4%. Inter-assay accuracy was between 99 and 102%, while precision was less than 2%. Recoveries for both analytes over the dynamic range were greater than 90% for all the tissues.

Keywords: Danofloxacin; N-Desmethyldanofloxacin; Antibiotics

1. Introduction

Danofloxacin, 7-[(1′s,4′s)-5′-methyl-2′,5′-diazabicyclo[2.2.1]hept-2′-yl]-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid Fig. 1 is a synthetic antibiotic of the fluoroquinolone class developed specifically for use in veterinary medicine [1]. Danofloxacin has been studied for use in cattle, swine, chickens, and turkeys for the control of respiratory and enteric bacterial infections [2–4]. It is highly effective against many Gram-positive and Gram-negative pathogens and has been shown to obtain a high tissue-to-plasma ratio [4]. In addition, the presence of a primary metabolite, N-desmethyldanofloxacin Fig. 1, has been identified in the edible tissues. The determination of both danofloxacin and N-desmethyldanofloxacin simultaneously was desired without interference of other fluoro-

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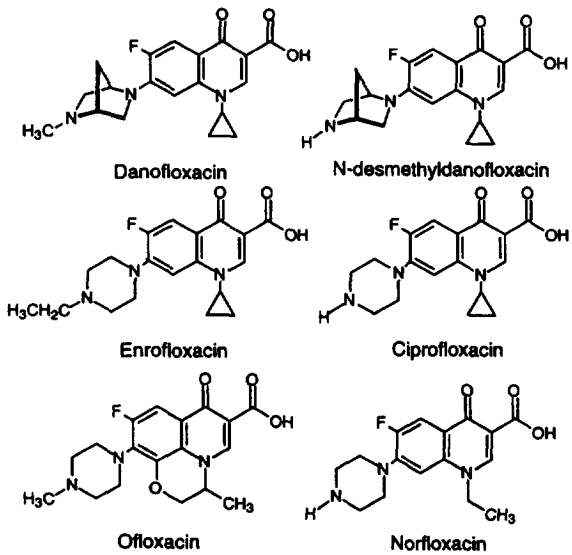

Fig. 1. The structures of the commonly used veterinary quinolones.

quinolones used in veterinary medicine. Among the fluoroquinolones on the market world wide enrofloxacin, ciprofloxacin, ofloxacin, and norfloxacin have been the most commonly used in veterinary medicine [5–8]. Therefore an analytical method was developed that has selectivity towards danofloxacin and N-desmethyldanofloxacin over these other fluoroquinolones.

Many of the original methods for fluoroquinolone analysis in tissue by liquid chromatography were hampered by laborious extraction procedures [9–11] or severely tailing peaks [12–14]. Most methods employed mixtures of various ion-pairing reagents in the mobile phase to increase selectivity and reduce peak tailing [13–17]. Recently, column manufacturers have produced higher-purity silica that is devoid of the trace metals and impurities that contributed to much of the peak tailing previously observed [20]. For the fluoroquinolones of interest, the Inertsil C_8 material demonstrated excellent peak shape, capacity, and resolution with a minimal mobile phase.

This paper describes a simple method for the determination of danofloxacin and N-desmethyldanofloxacin in chicken liver and muscle, cattle liver, muscle, kidney, and fat with selectivity over ofloxacin, norfloxacin, ciprofloxacin, and enrofloxacin. Selectivity is achieved through a combination of extraction, chromatography, and fluorescence detection. A liquid extraction of the fluoroquinolones is achieved using a mixture of methanol, perchloric and phosphoric acids. After precipitation of the proteins and cellular matter, direct injection of the supernate is possible.

2. Experimental

2.1. Reagents

The following reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) unless otherwise noted. Acetonitrile and methanol, HPLC grade. Monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), A.C.S. certified. Phosphoric acid, 86%, Baker analyzed. Perchloric acid, 70%. Water, 18.2 MΩ (HPLC grade), was produced using a Milli-Q water system, Millipore (Milford, MA, USA).

2.2. Sample preparation equipment

The following equipment was purchased from VWR Scientific (Boston, MA, USA) unless otherwise noted: Vortex-Genie mixer, Precision Scientific temperature-regulated water bath Model 83, Brinkman polytron homogenizer, with probe PT-10TS, and a Centra-8 centrifuge, International Equipment (Needham Hts, MA, USA).

2.3. Chromatography equipment

An Inertsil C_8 column, 250×4.6 mm I.D. (5 μm, 100 Å), packed by GL Sciences (Tokyo, Japan) or Column Engineering (Ontario, CA, USA) was used at a flow-rate of 1.0 ml/min provided by a ConstaMetric 4100 isocratic pump, LDC Analytical (Boca Raton, FL, USA). The column was maintained at a temperature of 35±0.5°C using a LC-22A column heater, Bioanalytical Systems (West Lafayette, IN, USA). A pre-column guard filter, 0.5 μm, Upchurch Scientific (Oak Harbor, WA, USA) was utilized to protect the column from small particles. A Shimadzu Model RF-10A fluorescence detector was utilized at an excitation wavelength of 280 nm and an emission wavelength of 440 nm. A Wisp 717 plus

autosampler with 200-μl glass vials (Waters, Milford, MA, USA) provided an injection volume of 20 μl. Data was acquired for 30 min per sample with a computer data acquisition system, MultiChrom 2, VG-Fisons (Beverly, MA, USA).

2.4. Solutions

Methanolic extraction solvent was made by combining 0.015 *M* $HClO_4$ and 0.015 *M* H_3PO_4 in water–methanol (50:50, v/v). One liter of phosphoric acid solution, 0.05 *M*, was prepared. HPLC mobile phase, 0.05 *M* phosphate buffer at pH 3.5 with 12% acetonitrile was prepared by dissolving 6.62 g of mono sodium phosphate ($NaH_2PO_4 \cdot H_2O$) into 900 ml of Milli-Q 18.2 MΩ water. The solution was adjusted to pH 3.5 using phosphoric acid. The solution was diluted to one liter, filtered, and degassed. The phosphate buffer (0.05 *M*) was added to 120 ml of acetonitrile, to make a total volume of 1000 ml.

2.5. Stock and standard curve solutions

Stock solutions of danofloxacin and N-desmethyldanofloxacin, 100 μg/ml, were prepared by dissolving approximately 1±0.5 mg of each compound into a separate vials. Then, 0.05 *M* H_3PO_4 was added by weight to the vial (assume 1 g=1 ml) for a final concentration of 100 μg/ml. A correction was made for activity of the individual lot utilized.

A secondary stock solution (combining both danofloxacin and N-desmethyldanofloxacin) at a concentration of 1 μg/ml was prepared. A 1-ml aliquot of 100 μg/ml danofloxacin and a 1-ml aliquot of 100 μg/ml N-desmethyldanofloxacin were pipetted into a 100-ml volumetric flask. The flask was filled to volume with 0.05 *M* H_3PO_4.

Standard curve solutions were prepared using the solutions above by weighing the secondary stock solution into a 10-ml volumetric flask (assume 1 g=1 ml) then diluting to volume with 0.05 *M* H_3PO_4. Process samples (fortified tissue) were prepared by fortifying the tissue with the stock solutions described above. The volume used for fortification of 0.5 g of tissue was 100 μl.

2.6. Sample preparation

Tissue samples were thawed by leaving sample containers shielded from light at room temperature for approximately 30 min. Then, 0.5±0.05 g of tissue was weighed into a disposable test tube. Next, 5±0.025 ml of extraction solvent was delivered into each tube. The tubes were mixed at high speed for 10 s. Each sample was homogenized for 30±10 s at high speed using the Polytron homogenizer. The Polytron blade was cleaned between samples by cycling for 15 s at high speed in (a) extraction solvent, then (b) water. All samples were mixed at high speed for 10 s to re-suspend the tissue in the extraction solvent. The sample tubes were capped and incubated in a 50±5°C water bath for 90±10 min. Following incubation, the tissue samples were centrifuged at 1200 *g* for 10 min at room tempera-

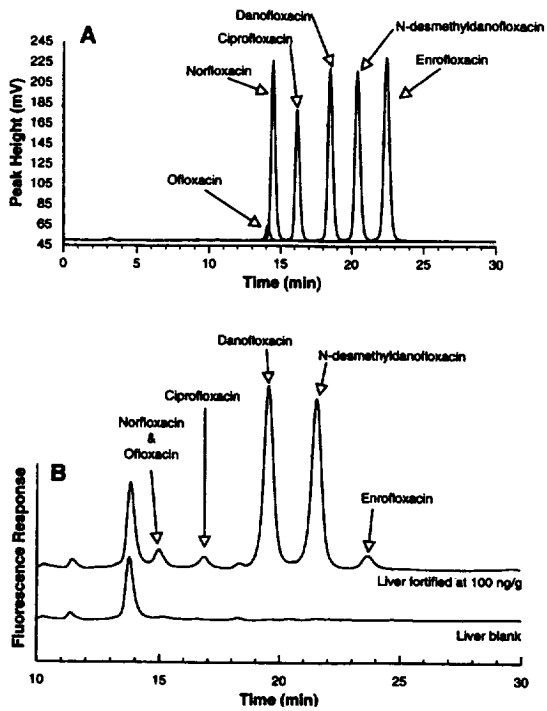

Fig. 2. (A) The composite chromatograms of the separation of ofloxacin, norfloxacin, ciprofloxacin, and enrofloxacin from danofloxacin and N-desmethyldanofloxacin. (B) Separation of ofloxacin, norfloxacin, ciprofloxacin, and enrofloxacin from danofloxacin and N-desmethyldanofloxacin in cattle liver fortified at 100 ng/g.

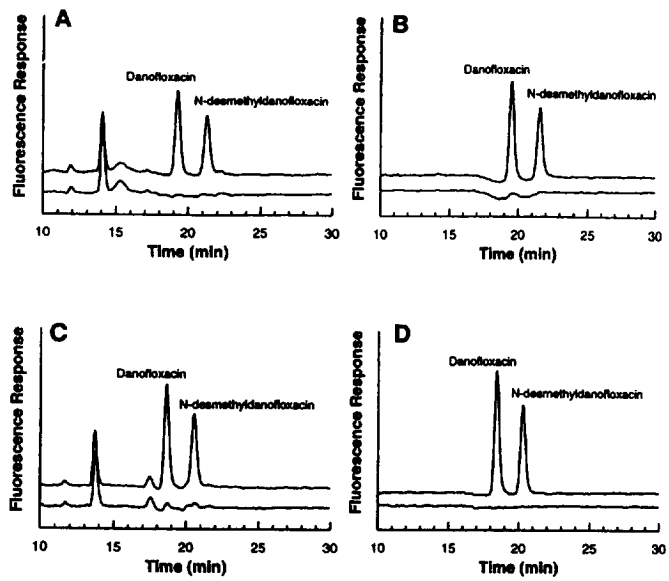

Fig. 3. Selectivity of the analysis over endogenous compounds in cattle liver, muscle, kidney, and fat. Danofloxacin elutes at 18.5 min, while N-desmethyldanofloxacin elutes at 20.5 min. Cattle tissues were fortified at 40 ng/g. (A) liver, (B) muscle, (C) kidney and (D) fat.

ture. Finally, 200 μl of supernatant was transferred into an autosampler vial.

Preparation of standard curve samples were achieved by pipetting 0.5 ml of each standard curve solution into a disposable test tube. Then, 5.0 ml of extraction solvent was added. All tubes were mixed at high speed for 10 s.

2.7. Calculations

The standard curve consisted of solutions at concentrations of 10, 20, 50, 100, 300, and 500 ng/ml. Concentrations of danofloxacin and N-desmethyldanofloxacin were independently calculated using the linear equation $y=mx+b$, where y was the peak height of analyte, and x was the analyte concentration. The values of the slope m and intercept b were obtained from the weighted linear least squares regression analysis of the intended concentrations of the standard curve solutions versus the corresponding peak heights. A weighting factor of $1/x$ was applied in the regression analysis.

3. Results and discussion

3.1. Chromatographic conditions

Fluoroquinolones are generally separated using ion-pairing agents to suppress peak tailing due to

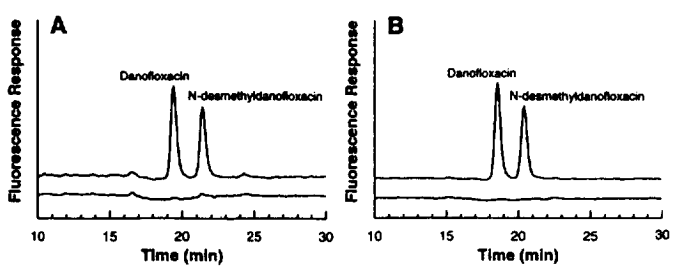

Fig. 4. Selectivity of the analysis method over endogenous compounds in chicken liver and muscle. Danofloxacin elutes at 18.5 min, while N-desmethyldanofloxacin elutes at 20.5 min. Chicken tissues were fortified at 40 ng/g. (A) liver, (B) muscle.

interactions with conventional reversed-phase columns [13–17]. Previous methods for danofloxacin analysis used a mixture of ion-pairing agents and counter ions to suppress interactions with the column packing material [15]. Residual silanol groups and metal impurities have been targeted as the major causes for the peak tailing [18,19]. Recently several manufactures, namely GL Sciences and Waters, have been providing highly end-capped reversed-phase column materials made with purified silica [20]. The amount of acidic trace metals, mostly Al and Fe, in this silica has been drastically reduced. The columns have resulted in a reversed-phase material that is inert enough under acidic conditions to provide excellent peak shape while maintaining capacity. Several recent publications have alluded to the use of this new type of column and used reduced amounts of ion-pairing agents [16] or completely eliminating them [21]. Our goal was to use the simplest separation method available, desiring an easy, rugged, and reproducible system. It was found that of the new types of columns available the Inertsil C_8 stationary phase resulted in a symmetry factor, calculated at 10% above baseline [22], of less than 1.2 for all fluoroquinolones tested. The capacity of this stationary phase was great enough to give the resolution desired in less than 30 min.

3.2. Selectivity and specificity

The structural similarity of the fluoroquinolones to be separated places a stringent requirement on the chromatographic method. Previous methods for the analysis of danofloxacin [15] were unable to resolve ofloxacin, norfloxacin, ciprofloxacin, and enrofloxacin from danofloxacin and N-desmethyldanofloxacin. This assay was designed to achieve baseline separation of all these molecules from danofloxacin and N-desmethyldanofloxacin. With the chromatographic system described, the retention times for danofloxacin and N-desmethyldanofloxacin are 18.5±0.5 min and 20.5±0.5 min, respectively. The method has shown selectivity for danofloxacin and N-desmethyldanofloxacin in the presence of norfloxacin, ofloxacin, ciprofloxacin, and enrofloxacin. Selectivity is achieved through a combination of

Table 1
Intra-day accuracy and precision values for the determination of danofloxacin and N-desmethyldanofloxacin

Compound	Concentration (ng/ml)	Mean (ng/ml)	Standard deviation	Accuracy (%)	Precision (%)
Danofloxacin	40	39.5	1.3	99	3
	100	101.1	1.5	101	1
	400	393.1	8.8	98	2
N-Desmethyldanofloxacin	40	39.8	1.5	99	4
	100	101.2	2.0	101	2
	400	392.8	9.0	98	2

$n=5$.

Table 2
Inter-day accuracy and precision values for the determination of danofloxacin and N-desmethyldanofloxacin

Compound	Concentration (ng/ml)	Mean (ng/ml)	Standard deviation	Accuracy (%)	Precision (%)
Danofloxacin	40	39.9	0.3	100	1
	100	100.2	2.0	100	2
	400	410.9	2.5	103	1
N-Desmethyldanofloxacin	40	39.8	0.4	99	1
	100	99.3	1.7	99	2
	400	406.0	2.8	102	1

$n=6$; three separate days run in duplicate.

Table 3
Recovery and precision values for the determination of danofloxacin and N-desmethyldanofloxacin in cattle and chicken tissues

Animal	Tissue	Danofloxacin						N-Desmethyldanofloxacin					
		40 ng/g		100 ng/g		400 ng/g		40 ng/g		100 ng/g		400 ng/g	
		% Recovery	% Precision	% Recovery	% Precision	% Recovery	% Precision	% Recovery	% Precision	% Recovery	% Precision	% Recovery	% Precision
Cattle	Liver	96	2	101	2	97	4	96	2	100	3	96	4
	Muscle	98	4	101	3	97	4	98	5	101	2	97	2
	Kidney	99	3	101	1	98	2	99	4	101	2	98	2
	Fat	107	3	111	1	108	1	109	2	112	1	108	1
Chicken	Liver	98	3	101	2	98	1	99	2	102	2	97	1
	Muscle	94	3	94	2	93	1	93	4	93	2	92	2

$n=5$, except cattle liver where $n=12$; triplicate samples on four days.

extraction, chromatography, and fluorescence detection. Fig. 2A illustrates the separation of the method for the fluoroquinolones of interest. Fig. 2B illustrates the separation of extracted cattle liver fortified at a level of 100 ng/g for all the fluoroquinolones of interest. Under the experimental conditions the peak heights are significantly greater for danofloxacin and N-desmethyldanofloxacin.

The method also exhibits selectivity for endogenous compounds, in cattle liver, muscle, kidney, and fat Fig. 3. No peaks were observed in the same chromatographic window as danofloxacin or N-desmethyldanofloxacin in the blank chromatograms that were greater than 5% of the lower limit of quantitation (10 ng/g) in three unique samples of each tissue. The same criteria also held true for chicken liver and muscle tissue Fig. 4.

3.3. Assay characteristics

This assay exhibits a linear dynamic range between 10 and 500 ng/g for both danofloxacin and N-desmethyldanofloxacin. A linear relationship is obtained across this dynamic range. The limit of quantitation was determined to be 10 ng/g (20 pg on column) based on a signal-to-noise ratio greater than 20.

The intra-assay accuracy and precision were determined using solutions at three concentrations on a single day. Samples were prepared to represent 40, 100, and 400 ng/g danofloxacin and N-desmethyldanofloxacin and assayed in replicate ($n=5$). The resulting intra-assay accuracy and precision values for each analyte are displayed in Table 1.

The inter-assay accuracy and precision was determined on three separate days using solutions at three concentrations covering the range of the assay. Samples were prepared to represent 40, 100, and 400 ng/g danofloxacin and N-desmethyldanofloxacin and assayed. The resulting inter-assay accuracy and precision values for each analyte are displayed in Table 2.

The recovery of danofloxacin and N-desmethyldanofloxacin from the tissues of interest was determined. The recovery of danofloxacin and N-desmethyldanofloxacin was determined by assaying fortified tissue samples at three concentrations (40, 100, and 400 ng/g). The determined concentration from a standard curve was divided by the intended concentration and the recovery established. The recovery values for cattle and chicken tissues are listed in Table 3. For the cattle liver samples, triplicate analyses of each sample were completed on four separate days. This provided for both intra- and inter-day variability characterization. Since no differences were seen between the intra- and inter-day analysis, for all of the other tissues, replicates of five at each of three concentrations were assayed on a single day. Recoveries for all samples were greater than 90%. Cattle fat consistently exhibited recoveries near 110% with very good precision, even though the blank samples did not present any interfering peaks. Additionally, the amount of fat used (when holding the amount of drug constant) did not affect the recovery of either danofloxacin or N-desmethyldanofloxacin. Several methods of homogenization were attempted without a correlation. This phenomenon is unexplainable at this time, as more studies are underway to identify a solution.

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