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Liquid chromatography analysis of enrofloxacin and ciprofloxacin in chicken blood spotted on filter-paper disks

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Abstract

A simple, low-cost, sensitive and selective LC method was developed for the determination of enrofloxacin and ciprofloxacin in chicken blood. The method was applied to whole blood from a chicken using dried blood spots on filter paper disks. The detection limits of enrofloxacin and ciprofloxacin (100 μl of whole blood on a disk) were 0.005 and 0.01 μg/ml, respectively. The whole procedure was verified in intra-laboratory studies (recoveries of both compounds were above 90%), and its applicability was tested with blood from the chicken receiving enrofloxacin in a single oral dose at a level of 10 mg/kg body mass. The method permits the use of a small volume of blood from a chicken and should be useful for pharmacokinetic studies.
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1. Introduction

Enrofloxacin (EFX) is a flouroquinolone (FQ) synthetic antibacterial agent with a broad spectrum of activity. The pharmacokinetics of EFX is characterized by good absorption and extensive distribution into various animal fluids and tissues. Disposition of EFX has been reported for numerous mammalian species as well as for fish and poultry [1–6]. In many animal species EFX is de-ethylated to ciprofloxacin (CFX), a potent antimicrobial agent, with a mechanism of action and antimicrobial activity similar to those of EFX [7,8].

Several liquid chromatographic (LC) methods have been developed for the determination of EFX and CFX in animal tissues and body fluids [9–14]. Some of them employ the solid-phase extraction (SPE) technique, which has the advantage of simplicity and accuracy. However this technique is quite expensive.

Pharmacokinetic studies require multiple blood sampling. However, collection of serial blood samples especially from as small animals as poultry is difficult or sometimes impossible to perform. The dried blood spot (DBS) method has been successfully used in diagnoses [15–18], and it has an advantage in terms of multiple sample collection from chickens.

The aim of this work was to develop a simple and low-cost method, which involves the DBS instead of SPE, and has a large dynamic concentration range in order for the determination of EFX and its metabo-

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lite, CFX, levels in chicken blood. We have validated the use of DBS and show that such sample taking can provide results comparable with those obtained after the use of SPE for FQ study in plasma.

2. Materials and methods

2.1. Reagents

Acetonitrile (ACN), trichloroacetic acid (TCA) and orthophosphoric acid, LC and analytical grade, were from Merck (Darmstadt, Germany). 1-Heptanesulfonic acid was obtained from Sigma (Poole, UK). EFX was from Union Quimico Farmaceutica (Barcelona, Spain) and CFX was from Flavine (Madrid, Spain). The cartridges Bakerbond octadecyl (500 mg) were purchased from J.T. Baker (Deventer, The Netherlands), and S&S antibiotic-assay discs, (diameter 12 mm) were from Aldrich (Dassel, Germany).

2.2. Preparation of standard solutions and spiked samples

2.2.1. Analytical standard solutions

Stock solutions of EFX and CFX (1 mg/ml) were prepared in acetonitrile. Working standards (5 and 10 μg/ml) were prepared by dilution with LC mobile phase.

2.2.2. Spiking standard solutions

Spiking standard solutions (25 and 50 μg/ml) were prepared from stock solutions by dilution with acetonitrile–water (1:1, v/v).

2.2.3. Preparation of spiked DBS

Citrated whole blood from healthy adult broiler chicken without previous treatment was used. Spiked chicken blood standards ranging from 0.01 to 5 μg/ml were prepared from the spiking standard solutions and drug-free whole blood.

DBSs were prepared by apply drug-free blood or blood spiking standard to the center of the filter paper circle. The spots were dried at room temperature (10 min) and were stored in air-tight plastic bags. The DBSs with drug-free blood and spiked blood standard were kept at −20 °C until the use to optimise elution conditions and validation studies.

2.3. Liquid chromatography

A Shimadzu VP Series liquid chromatograph (Duisburg, Germany) equipped with a degasser and a mixer of mobile phase was used. A fluorescence detector FR-10AXL with an excitation wavelength of 278 nm and emission wavelength of 440 nm was used to analyze the tested solutions. LC control, data acquisition and peak integration was performed by the system controller SCL-10A utilizing the RS-232C interface for communication with the CLASS-VP chromatography workstation.

The chromatographic analyses were performed on a LiChrospher 100 RP-8, 5 μm (250×4.6 mm) column. The mobile phase for LC analyses consisted of ACN–solution of 2.5 n*M* orthophosphoric acid, pH 3.0 containing 2.5 n*M* of 1-heptanesulfonic acid (70:30, v/v), both were filtered before use. A flow-rate of 1.0 ml/min was used for the separation of analytes at ambient temperature. Aliquots from 20 to 100 μl were injected into the column.

2.4. Optimisation of drug isolation from DBSs

Spiked DBSs were dipped in glass tubes with different volumes (200–1000 μl) of solvent. The efficiency isolation of EFX and CFX was checked by monitoring the recovery of EXF and CFX as a function of time, by gently shaking the tubes or by ultrasonication.

2.5. Method comparison study

A group of broiler chickens ($n=6$) was treated with a single oral dose of enrofloxacin at a level of 10 mg/kg body mass (b.w.). Blood was taken by venipuncture from the right vein at selected intervals after administration: 0, 0.5, 1, 2, 3, 6, 4, 8, 10, 12 and 24 h.

2.5.1. Blood spot preparation

Blood was collected directly onto the circle so that the filter paper was filled on both sides. The spots were labeled with the date and time of collection, allowed to dry at room temperature and were stored in air-tight plastic bags at +4 °C until assay.

Table 1
Influence of solvents on the extraction of EFX and CFX from DBSs

Solvent	Recovery (%)	
	EFX ($n=6$)	CFX ($n=6$)
Water	0.00	0.00
0.9% saline	5.75	2.59
5% TCA	10.45	9.75
Phosphate buffer, pH 7.5	8.56	5.90
Methanol	50.65	35.95
60% Ethanol	70.36	65.32
Acetonitrile	95.43	92.85

2.5.2. Assay of plasma samples

The same venous blood was placed in a collection bottle containing heparin anticoagulant, centrifuged and the plasma was stored at −20 °C until assay. Levels of EFX and CFX were measured after deproteinisation of plasma samples with a trifluoroacetic acid–acetonitrile (3:7) mixture, and clean up by SPE with an octadecyl cartridge as described previously [9].

3. Results

3.1. Optimising elution from DBSs

The extraction efficiency was performed with distilled water, physiological saline, organic solvents and protein precipitant (Table 1). The optimum elution of EFX and CFX was checked with 500 μl of acetonitrile. It was found that 30 min of ultrasonication ensured good recoveries of EFX and CFX (Table 2), and also denatured the protein in the eluates.

Table 2
Efficiency of isolation methods for EFX and CFX recovery (%) from DBS ($n=6$) with 500 μl of acetonitrile

Time (min)	Shaking		Ultrasonication	
	EFX	CFX	EFX	CFX
5	15.5	12.5	20.8	21.5
10	20.8	15.2	22.7	23.0
15	35.3	36.8	48.3	53.0
30	60.3	58.3	96.3	95.2
45	71.5	72.4	96.0	95.4
60	72.3	70.4	95.8	92.3

3.2. Assay reproducibility

To obtain information on the volume of blood and the reproducibility of blood collection, the studies were performed as described previously [15,16]. The DBSs with drug-free blood were extracted with 0.5 *M* disodium phosphate solution and the eluates were measured by spectrometrically based on the absorption of the haemoglobin at 575 nm. The blood volume in S&S antibiotic-assay filter paper discs was found to average 100 μl with a standard deviation (SD) of 0.37 ($n=6$). The intra-assay relative standard deviation (RSD) for the blood volume in 12 discs was 4.3%.

3.3. Validation study

3.3.1. Selectivity

Under the assay conditions described above, EFX and CFX were well resolved with retention times of 5.3 and 7.9 min, respectively (Fig. 1a). The method exhibits selectivity for endogenous compounds, in chicken blood samples. No interfering peaks were observed in the same chromatographic windows as CFX or EFX in the blank chromatogram (Fig. 1b–d).

3.3.2. Analytical recovery and precision

The extraction recovery of EFX and CFX from chicken blood was measured at three concentrations, namely, 0.05, 0.50 and 2.5 μg/ml. The recoveries of EFX and CFX from spiked DBS samples were calculated by comparison with a solution of suitable analyses. Both the FQs were eluted with high efficiency, above 90%.

Table 3 shows the intra-assay and inter-assay results of this method.

3.3.3. Linearity

The linearity of the assay was checked using DBS spiked with the working solutions, to final concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.5 and 5.0 μg/ml. The correlation coefficients were 0.9978 and 0.9986 for EFX and CFX, respectively.

3.3.4. Limit of detection and determination

The limits of detection in DBS, using a signal-to-

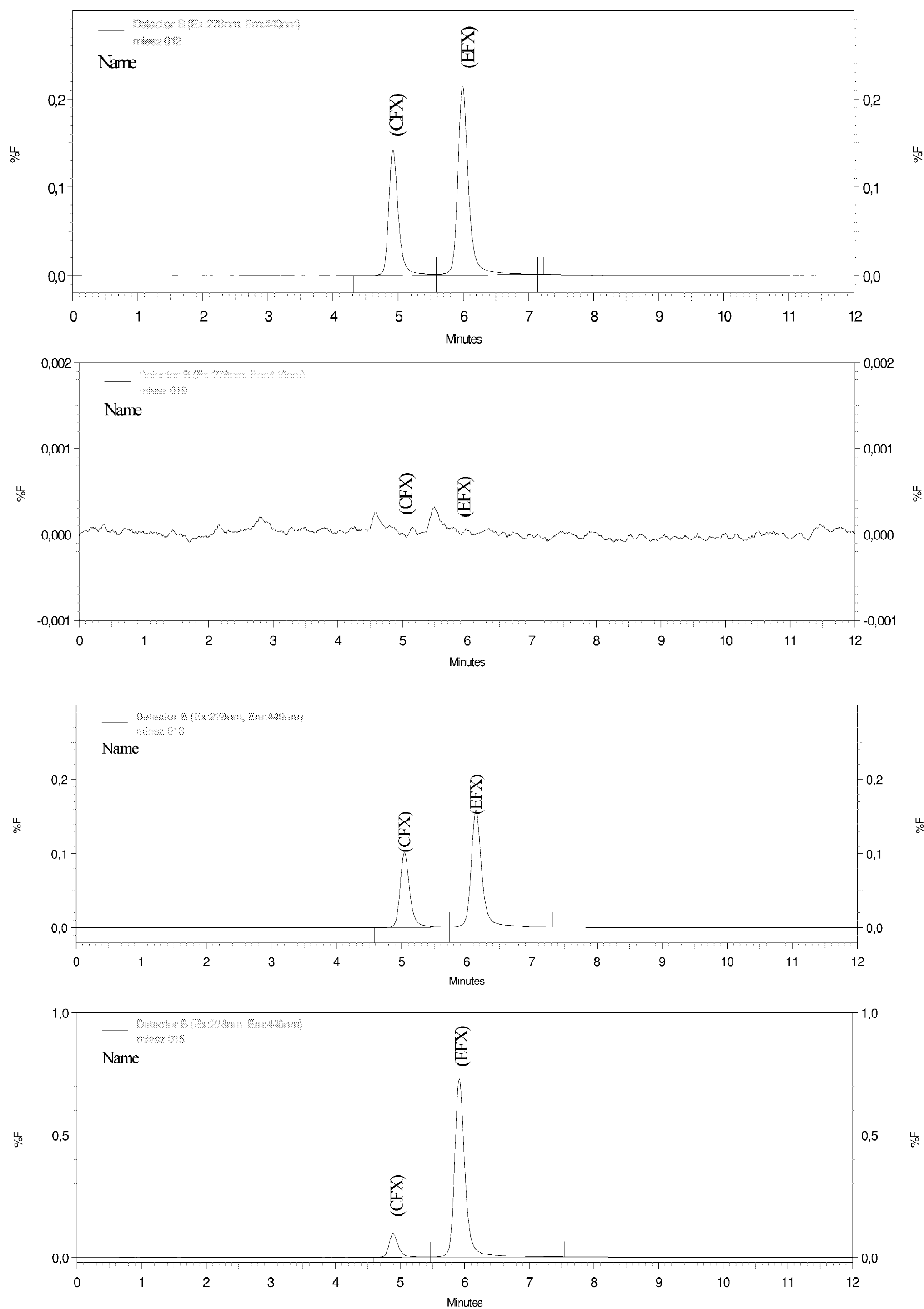

Fig. 1. Typical chromatograms of DBS extracts of chicken blood. (a) Standard mixture. Peaks: CFX=20 ng; EFX=10 ng. (b) Drug-free chicken blood DBS extract. (c) DBS extract of chicken blood spiked with 2 μg/ml of CFX and 1 μg/ml of EFX. (d) DBS extract from the treated chicken after oral administration of 10 mg/kg of EFX.

Table 3
Analytical recoveries of EFX and CFX from DBS

Drug	Added (μg/ml)	n	Determined (mean±SD) (μg/ml)	Recovery (%)	RSD (%)	
					Intra-assay	Inter-assay
EFX	0.05	6	0.047±0.004	94.6	4.3	5.3
	0.50	6	0.463±0.007	92.7	5.8	6.4
	5.00	6	4.590±0.008	91.8	3.4	6.1
CFX	0.05	6	0.046±0.006	92.8	5.3	5.6
	0.50	6	0.458±0.005	91.7	5.1	6.2
	5.00	6	4.651±0.004	93.5	4.5	6.8

noise ration 2:1, were 0.005 and 0.010 μg/ml for EFX and CFX, respectively. The limits of determination were established at the level of 0.05 μg/ml for EFX and CFX.

3.4. Stability study

The stability of EFX and CFX in DBS during storage at −20, +4 °C and room temperature was determined. A series of blood spots with EFX and CFX at the level of 1 μg/ml were stored for 4 weeks at the above conditions. The concentrations were determined on the day of the DBS preparation and after storage for 4 weeks. A statistical paired Student's t-test ($\alpha=0.05$) showed that the concentrations were not significantly different after storage, indicating that EFX and CFX are stable in a filter-paper matrix for 4 weeks.

3.5. Comparison methods

The concentrations of EFX and CFX versus time determined by DBS or plasma assay are shown in Table 4.

In the plasma assay, the EFX concentrations peaked 2 h after oral administration (C_{max}= 0.98±0.09 μg/ml) and then declined to 0.02±0.01 μg/ml 24 h after dosing. In the DBS assay, the highest concentrations were obtained after 1.5 h (C_{max}=1.16±0.08 μg/ml) from the oral dosing and 0.02±0.02 μg/ml after 24 h.

Ciprofloxacin concentrations rose rapidly after dosing and remained above the detection limits of both the procedures for at least 12 h.

As shown in Table 4 the concentrations obtained with the two methods were in good agreement, with a linear correlation. For EFX, the equation of the fitted curve was $y=1.8x-0.03$ (n=20, r=0.98). A similar excellent correlation was obtained for CFX.

Table 4
Comparison of EFX and CFX concentrations assayed by plasma procedure and DBS from treated chickens (single dose of 10 mg/kg b.w.)

Time after treatment (h)	DBS		Plasma assay	
	EFX	CFX	EFX	CFX
0.5	0.45±0.02	0.01±0.02	0.34±0.08	0.02±0.02
1.0	0.86±0.09	0.04±0.03	0.76±0.09	0.03±0.02
2.0	1.17±0.11	0.04±0.02	0.98±0.10	0.04±0.02
3.0	0.89±0.07	0.02±0.02	0.88±0.05	0.03±0.02
4.0	0.72±0.06	0.02±0.01	0.65±0.06	0.03±0.01
6.0	0.52±0.05	0.03±0.01	0.53±0.07	0.02±0.02
8.0	0.22±0.07	0.03±0.02	0.19±0.03	0.02±0.02
10.0	0.17±0.06	0.01±0.01	0.15±0.03	0.01±0.02
12.0	0.10±0.05	0.01±0.01	0.10±0.03	0.01±0.01
24.0	0.02±0.01	nd	0.02±0.01	nd

4. Discussion

Samples on filter paper have been used for clinical purposes or control of therapeutic levels of theophyline, phenytion and aminoglycoside antibiotics [15–18] but this is the first report, to our knowledge, of its application to FQ estimation.

The methodology presented has combined the advantage of microsampling, inherent in blood collection on filter-paper, with an accurate, precise and selective LC technique. This simple method shows linear correlation between EFX and CFX concentrations in plasma and blood spots. Moreover, the sensitivity of the LC for EFX and CFX in filter-paper spots was comparable with that of LC for EFX and CFX in plasma, confirming its validity.

The concept of monitoring the EFX and CFX concentrations in blood using the DBS technique arises from the practical problems of obtaining samples from small animals like birds, without serious damage. In our opinion this blood-spot technique is suitable for FQ determination in blood samples, a definitive time–course assay for pharmacokinetic studies of FQs in individual chicken or other small animals is feasible. The technique minimizes the need to provide facilities for storing blood samples in the laboratory.

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