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High-performance liquid chromatographic assay and erythrocyte partitioning of fleroxacin, a new fluoroquinolone antibiotic

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Abstract: A rapid technique for sample preparation and HPLC was developed to study *in vitro* the erythrocyte binding and plasma protein binding of fleroxacin in the concentration range 0.2–15 μg ml^{-1}. The red blood cell partition coefficient of fleroxacin was 1.45 ± 0.18 at 25°C and was independent of concentration, incubation time and temperature. The *in vitro* plasma protein binding of fleroxacin in humans, determined by two independent methods, was 47–51%. Partition studies using erythrocytes as a simple *in vitro* model may be useful to predict the degree of tissue uptake and the volume of distribution.

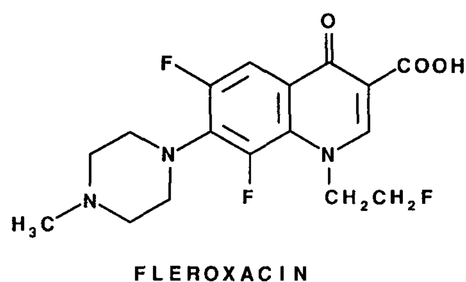
Keywords: *Fleroxacin; HPLC assay; stability; red blood cell binding; plasma protein binding.*

Introduction

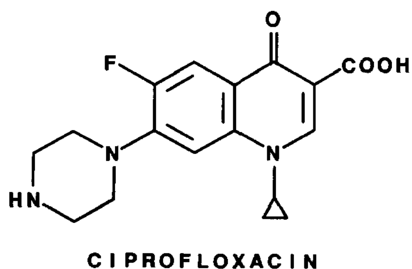
Fleroxacin is a new fluoroquinolone antimicrobial agent, which has a broad spectrum of activity against gram-positive and gram-negative bacteria, such as Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilis influenzae* and Staphylococci [1, 2]. The efficacy of fleroxacin against many systemic as well as local infections may be a result of its high oral bioavailability [3] and high concentrations in tissue [2].

Preliminary human pharmacokinetic studies of fleroxacin indicated that it has a longer half-life than other quinolone antimicrobial agents [4]. After oral administration of a 400-mg dose of fleroxacin to human subjects, the mean peak level in serum was 5–6 μg ml^{-1}, which was attained 0.7–1.3 h after administration, and the elimination half-life was about 12 h [1]. The *in vitro* protein binding of fleroxacin in humans, determined by equilibrium dialysis, was reported to be 23% [3]. The *in vivo* protein binding determined by ultrafiltration was 41–57% in rats and 41–61% in dogs at total concentrations of 1–7 μg ml^{-1} [5].

The red blood cell (RBC) partitioning of fleroxacin has not yet been studied. Information on erythrocyte binding may be important since all drugs that are distributed to their target sites in the body via the systemic circulation come in contact with blood cells. In previous studies [6] it has been shown that the degree of drug binding to erythrocytes can be described by a red-blood-cell-partition coefficient *D*. For example, for a series of cephalosporins, *D* was reported to be very small indicating very little RBC-uptake or binding for this class of antibiotics [6].



FLEROXACIN



CIPROFLOXACIN

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The purpose of this study was to investigate the RBC partition coefficient of fleroxacin and to compare it with that of another fluoroquinolone, ciprofloxacin. Further objectives were to study a possible time and temperature dependency of the RBC partitioning of fleroxacin, and to compare partition studies in erythrocyte suspensions with partition studies in whole blood, which allows the determination of the plasma protein binding.

To achieve these objectives it was first necessary to develop a sensitive and reproducible analytical technique to quantify fleroxacin in plasma, plasma water and whole blood.

Materials and Methods

Materials

Fleroxacin and ciprofloxacin were provided by Hoffmann-La Roche Inc. Research Center (Nutley, NJ) and Bayer (Leverkusen, FRG). All other chemicals were either USP (US Pharmacopeia), NF (National Formulary) or ACS (American Chemical Society) quality and were used without further purification. Solvents for the mobile phase were HPLC grade except the phosphoric acid solution, which was ACS certified. Human blood was obtained from the Civitan Regional Blood Center (Gainesville, FL).

Apparatus

For the HPLC assay the following instruments were used: high-pressure pump, Constametric III G, LDC/Milton Roy (Riviera Beach, FL); a LC fluorometer, FS 970 with a monochromator GM 970, Kratos Analytical Instruments (Ramsey, NJ); an octadecylsilane (ODS) column, Zorbax ODS (5 μm) 150 × 4.6 mm i.d., Du Pont Instruments (Wilmington, DE) with a guard column packed with Zorbax ODS 40 × 4.6 mm, Du Pont Instruments (Wilmington, DE); a strip-chart recorder, series 5000, Fisher recordall, Fisher Scientific Co. (Fair Lawn, NJ); an injector, Negretti & Zombra Ltd (Southampton, England); and a 100-μl syringe, Hamilton Company (Reno, NV).

In order to filter the mobile phase a Teflon filter membrane (Lazar Scientific, Los Angeles, CA) was used.

For the preparation of the UV and fluorescence spectra a UV–vis. spectrophotometer Lambda 3B, Perkin–Elmer (Oak Brook, IL) connected with a recorder R100A, Perkin–Elmer and a fluorescence spectrophotometer LS-5, Perkin–Elmer connected with a graphics printer GP-100, Perkin–Elmer were used.

A laboratory centrifuge (Dynac II centrifuge, Clay Adams) was used to separate plasma or buffer from RBCs and to separate aqueous phase from organic extracts.

In order to determine the protein binding, Centricon centrifugal microconcentrators (Amicon, Danvers, MA) were centrifuged in a Model J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA).

The haematocrit was determined, using microhaematocrit capillary tubes, Allied Corporation, Fisher Scientific (Pittsburg, PA) and a microcentrifuge IEC MB centrifuge, Damon/IEC Division (Needham HTS).

Chromatographic conditions

All samples were assayed by HPLC using a Zorbax ODS column. The mobile phase comprised 0.025 M phosphoric acid (adjusted to pH 2.4 with 0.002 M tetrabutylammonium hydroxide)–methanol–acetonitrile (88:11:1, v/v/v). The mobile phase was filtered through a Teflon filter membrane and degassed before use. The flow rate was 1.5 ml min^{-1} and a fluorescence detector (excitation wavelength 281 nm; 470-nm band pass emmision filter) was used. The chart speed was 0.25 cm min^{-1}. All assays were performed at room temperature. For the analysis of fleroxacin, ciprofloxacin was used as the internal standard and vice versa. The retention times of fleroxacin and ciprofloxacin under these conditions were 5.2 and 8.8 min, respectively.

Partition studies in RBC suspensions

Human blood was centrifuged for 15 min at 1500 rpm. The plasma was removed and isotonic saline solution was added to the erythrocytes. The RBCs were gently suspended and centrifuged for 10 min at 1500 rpm. This washing procedure was repeated three or four times to remove all plasma proteins. RBC suspensions were spiked with the drug dissolved in modified isotonic phosphate buffer (3.72 g KH_2PO_4 and 16.18 g Na_2HPO_4 in 1.0 l of deionized water) to yield total drug concentrations of 0.2–12.0 μg ml^{-1} and an haematocrit of about 0.3. The haematocrit was determined routinely for each sample, using a microcentrifuge with capillary tubes. The pH was controlled before and after ad-

dition of the drug. The spiked suspensions were incubated for 5–30 min including a centrifugation time of 5–10 min at 1500 rpm.

For each concentration a reference solution, without erythrocytes, but with the same volume and the same amount of drug was treated in the same way. An aliquot of the supernatant solution as well as the reference was spiked with internal standard and analysed using the HPLC procedure. The RBC partition coefficient was calculated, using equation (1),

$$D = \frac{C_{ref} - C_{pw}(1 - H)}{C_{pw} \cdot H}, \quad (1)$$

where C_{ref} is the concentration in the reference solution, C_{pw} is the concentration in plasma water and H is the haematocrit. The RBC partition coefficient (D) of fleroxacin and ciprofloxacin was determined at 25 and 37°C.

Plasma protein binding studies using ultrafiltration

Plasma (2.0 ml) was spiked with different amounts of fleroxacin to give plasma concentrations of 2.5–15.0 $\mu g\ ml^{-1}$. The plasma was placed in a Centricon centrifugal concentrator with a low-adsorption, hydrophilic, YM membrane, with a 10,000 molecular-weight cut-off. The centrifugation time was 15 min at 6000 rpm, which gave a filtrate of about 0.3 ml. The filtrate (0.2 ml) was mixed with 0.2 ml of a ciprofloxacin solution (internal standard) and injected into the HPLC system. The filtrate of a plain plasma solution was analysed to make sure that there were no interfering peaks from other plasma compounds. Different solutions of fleroxacin in isotonic phosphate buffer (1–20 $\mu g\ ml^{-1}$) were partly filtered through the membrane to see if fleroxacin was adsorbed on the membrane. The unfiltered as well as the filtered part were analysed. The fraction of the drug bound to proteins can be calculated using the following equation:

$$f_b = \frac{C_{tot} - C_{free}}{C_{tot}}, \quad (2)$$

where C_{tot} is the total drug concentration and C_{free} is the unbound drug concentration. Free concentrations were corrected for the small degree of membrane binding.

Partition studies in whole blood

Whole blood (5.0 ml) was spiked with different amounts of the drug to give whole blood concentrations of 0.2–12 $\mu g\ ml^{-1}$. The blood was allowed to incubate for 30 min at room temperature, including a centrifugation time of 10 min at 2000 rpm. The supernatant plasma was analysed by the following procedure. The plasma was spiked with internal standard and NaOH was added. Plasma (2.0 ml) and acetonitrile (6.0 ml) were mixed and vortexed for 5 s. The mixture was centrifuged for 20 min at 2600 rpm. Of the resulting supernatant, 4 ml was extracted with 4 ml of chloroform–butan-1-ol (3:1, v/v), vortexed for 10 s and centrifuged for 5 min at 2600 rpm; then 0.4 ml of the resulting aqueous supernatant was evaporated under nitrogen, dissolved in 0.4 ml 0.002 M NaOH and injected into the HPLC system. A calibration curve of the drug in plasma over the range of 0.2–20 $\mu g\ ml^{-1}$, treated in the same way as described above, was prepared.

Results

HPLC assay

The conditions allowed simple and reproducible quantification of fleroxacin and ciprofloxacin in biological fluids. As it is difficult to extract these amphoteric drugs into organic solvents a different clean-up procedure was used where, after deproteinization, the interfering plasma components were extracted into chloroform–butanol, leaving the compounds of interest in the aqueous plasma phase. Calibration curves were linear over a range

Table 1
Inter-day variability of plasma analysis

Concentration of fleroxacin ($\mu g\ ml^{-1}$)	Concentration found ($\mu g\ ml^{-1}$)			Mean	SD*	RSD†
1.03	1.01	1.09	1.11	1.07	0.05	4.9%
8.24	8.47	8.37	8.48	8.44	0.06	0.7%
12.36	12.12	12.69	12.81	12.54	0.37	2.9%

*SD = standard deviation.
†RSD = relative standard deviation.

from 0.2–20 μg ml^{-1}. Inter-day precision was better than 5% (Table 1) and the mean error in the investigated concentration range was 3.4%. The limit of detection was 0.1 μg ml^{-1},

Stability of fleroxacin

In aqueous solution fleroxacin degraded rapidly in the presence of light. When protected from light, stock solutions in 0.002 M NaOH were stable at 5°C for at least 2 weeks. The same solution exposed to light degraded with a half-life of 25 h (Fig. 1). Also, when protected from light, fleroxacin was stable in plasma for at least 24 h at room temperature.

Erythrocyte binding

The RBC partition coefficient (D) for fleroxacin was 1.45 ± 0.18 at 25°C and 1.30 ± 0.24 at 37°C in RBC suspensions with fleroxacin concentrations of 0.25–12.36 μg ml^{-1}. The mean D of ciprofloxacin was slightly greater than that of fleroxacin. Figure 2 indicates that D for fleroxacin is independent of concentration and temperature for the investigated ranges. The RBC partitioning of fleroxacin and ciprofloxacin was much greater than that of several cephalosporins (D = 0.05) [6]. The D of fleroxacin was independent of the incubation time for incubation times of 5–30 min.

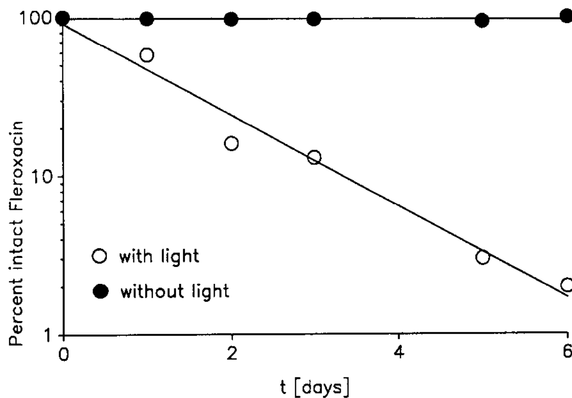

Figure 1
Stability of an aqueous fleroxacin stock solution in 0.002 M NaOH.

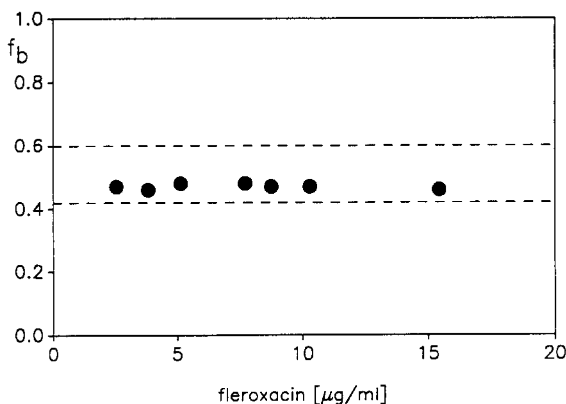

Figure 2
Protein binding of fleroxacin expressed as the fraction bound (f_b) to plasma proteins. The dotted lines indicate the range for results obtained by partition studies in whole blood; the points are results from studies using ultracentrifugation.

Protein binding determined by ultracentrifugation

The protein binding of fleroxacin was 47.0% ± 0.8% by ultrafiltration (Fig. 2). These results are in contrast to data previously reported in humans [3], but agree with some studies in dogs and rats [5]. No concentration dependence of plasma protein binding was observed.

Blood plasma ratios

The mean ratio between fleroxacin concentrations in blood and in plasma was found to be 0.86 ± 0.06. No concentration dependence of this ratio was seen. Hence, there is only a small difference of about 10% between plasma and blood levels due to the distribution of fleroxacin between plasma proteins and RBCs. For some cephalosporins it has been shown that plasma levels were almost twice the corresponding blood levels.

Protein binding calculated from drug distribution in whole blood

For comparison with the results obtained in the ultrafiltration studies, the degree of protein binding was also determined from partition studies in whole blood. This is possible since only the free, non-protein-bound drug is able to interact with the blood cells. The blood/plasma ratio can be used to calculate the fraction of drug bound to plasma proteins by using equation (3):

$$f_b = \frac{(C_b/C_p) - 1 + H + D \cdot H}{D \cdot H}. \quad (3)$$

The mean fraction bound was calculated to be 0.51 ± 0.09 (Fig. 2). This is in close agreement with the results observed in the ultrafiltration studies.

Discussion

Erythrocytes provide a simple laboratory model to study the uptake or binding of drug into cells. It is possible that RBC binding may be useful to predict tissue uptake of these drugs. The pharmacokinetic parameter describing the degree of tissue binding is the volume of distribution (V_d). The more drug penetrates into the tissue, the lower the plasma concentration and the higher the volume of distribution. Table 2 shows a comparison of RBC partition coefficients and volumes of distribution for several cephalosporins and the quinolones investigated in this study. For the cephalosporins V_d is close to the volume of extracellular water, indicating very little, if any, uptake of drug into the tissue cells. For the quinolones V_d is clearly larger than the total body water indicating some degree of cell uptake. At the same time RBC partition coefficients are very low for the cephalosporins and much higher for the quinolones. It is obvious that no general conclusions can be made based on the limited available data. Drugs can have different binding mechanisms to erythrocytes such as specific binding (e.g. acetazolamide) or non-specific binding to different cellular structures. Hence, more studies on more compounds are needed to verify this correlation.

Table 2
Comparison of D and Vd_{ss} of some cephalosporins and quinolones

	D*	Vd_{ss}† (1)
Cefotaxime	0.11	27
Ceftazidime	0.09	16
Cephalexin	<0.02	15
Cefazolin	0.08	10
Cefoxicin	0.06	12
Fleroxacin	1.45	98
Ciprofloxacin	1.54	184

* D = erythrocyte partition coefficient.
† Vd_{ss} = volume of distribution at steady state.

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