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Journal of Chromatography B, 703 (1997) 279–283

JOURNAL OF
CHROMATOGRAPHY B

Short communication

Rapid and simple determination of fleroxacin in rat plasma using a solid-phase extraction column

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Received 30 December 1996; received in revised form 10 June 1997; accepted 4 August 1997

Abstract

We studied the use of high-performance liquid chromatography (HPLC) with a spectroflurometric detector, using a solid-phase extraction column (Bond Elut cartridge column), for the simple, rapid and sensitive determination of plasma fleroxacin (FLRX) levels in rats. Extracted aliquots were analyzed by HPLC, using a reverse phase octadecyl silica column. The analytical mean recovery of FLRX added to the blank plasma averaged 101.4%. The detection limit was 58 ng/ml in the plasma. The reproducibilities (C.V.) were 0.50–3.22% in the within-day assay and 2.87 C.V.% in the between-day assay, indicating that the analysis method was effective in the determination of FLRX plasma levels. © 1997 Elsevier Science B.V.

Keywords: Fleroxacin

1. Introduction

Fleroxacin (FLRX), 6,8-difluoro-1-(2-fluorethyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolonine carboxylic acid, has 3 fluorine atoms attached to the quinolone system and is a new member of the class of fluoroquinolones. Preliminary reports indicated that this compound has a broad and potent antibacterial activity against Gram-negative and Gram-positive bacteria and that it is well absorbed after oral administration in various experimental animals [1,2]. This drug is often used in combination with other drugs [3].

To evaluate the pharmacokinetic interactions among these drugs [4,5] in experimental animals, a simple method for the measurement of the drug concentrations in the plasma is desired. The published methods for the determination of plasma FLRX in rats [6] have a number of drawbacks. Also much time is required during the analysis to condense and dry the extract obtained using chloroform, dichloromethane and acetonitrile [6–8].

Accordingly, for the investigation of the pharmacokinetics of FLRX, we have developed a method for the determination of FLRX in rat plasma. It

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PII S0378-4347(97)00409-X

requires only small plasma samples and uses a solid-phase extraction column for easy pretreatment.

2. Experimental

2.1. Drug

FLRX was kindly donated by Kyorin Pharmaceutical Co., Ltd. (Japan). Salicylamide was used as an internal standard (I.S.). Methanol was for high-performance liquid chromatography (HPLC) and other reagents were of analytical grade.

2.2. Animals

Twenty-two male Sprague–Dawley rats (250–310 g) were housed in plastic walled cages (26×36×25 cm; three or four animals per cage), and had unlimited access to food and water except for 12 h before and during the experiment. The animals were maintained on a 12-h light/12-h dark cycle (light on from 8:00 to 20:00). The ambient temperature and humidity were kept at 22–24°C and ca. 60%, respectively.

2.3. Apparatus for determining the plasma drug level

For plasma separation from the blood sample, a hematocrit centrifuge Hemac CT12 Hitachi was used. A spectrophotometer RF540 Shimadzu was used for selecting the absorption wavelength to detect FLRX in plasma. The drug plasma concentrations were determined by high-performance liquid chromatography (HPLC) (Shimadzu LC4A) with a spectrofluorometric detector (RF-10A, Shimadzu), and were calculated using a data module (Chromatopac C-R2AX, Shimadzu). A stainless-steel column packed with octadecyl silica (Shim-pack CLC-ODS, 150 mm×6.0 mm I.D., 5 μm particle size Shimadzu) was maintained at 40°C. The mobile phase was methanol–1 m*M* $(C_4H_9)_4HPO_4$ in 50 m*M* NaH_2PO_4, pH 3.0 (30:70, v/v) and the flow-rate was 1.0 ml/min. Measurements were made at an excitation wavelength of 290 nm and emission wavelength of 450 nm with salicylamide as an I.S.

2.4. Pretreatment of plasma for drug determination

For the extraction of FLRX and the I.S., a Bond Elut (1 ml volume, No. 070118 Analytichem International) solid-phase extraction (SPE) column containing octadecylsilica was used. The column was prewashed twice with 1 ml of methanol, followed by two washings with 1 ml of distilled water. Then 500 μl of 50 m*M* NaH_2PO_4 (pH 3.0), 20 μl of plasma containing FLRX and 30 μl of 20 μg/ml I.S. solution were added to the column. Two 1-ml portions of distilled water and 0.5 ml of 50 m*M* NaH_2PO_4 were passed through the column, then FLRX and the I.S. retained on the column were eluted with 500 μl of methanol–50 m*M* NaH_2PO_4 (pH 3.0, 90:10). The time necessary for pretreatment of plasma for drug determination was approximately 7 min. A 5-μl volume was used for injection into the HPLC system. This volume of methanol did not affect the shape of the chromatogram. The chromatographic peaks of FLRX and the I.S. from plasma samples were coincident with those of authentic standards.

2.5. Calibration curve

FLRX solutions of 125, 250, 500, 1000, 2000, 4000, 8000 ng/ml were prepared by dilution of the stock standard with distilled water. These solutions were injected into the HPLC apparatus after the same pretreatment as for drug-free plasma. The calibration curve for FLRX concentration was made from the peak-area ratio of FLRX and the I.S.

2.6. Precision

To investigate the precision of plasma FLRX measurements, plasma samples obtained from the cervical vein were analyzed ten times. Correlation between plasma samples obtained from the cervical vein and tail vein.

Blood samples from the tail vein as well as the cervical vein obtained under laparotomy were taken 0.5 and 1 h after FLRX was administered orally at a dose of 10 or 20 mg/kg. Plasma was separated by centrifugation, and the correlation between the two sets of samples was determined as described above.

2.7. Concentration–time profile of plasma FLRX

To determine the concentration–time profile of plasma FLRX, blood samples were obtained from the tail vein at 0.25, 0.50, 1.00, 2.00, 3.00, 4.00, 8.00, 12.00 and 24.00 h following oral administration of FLRX at doses of 10 and 20 mg/kg. The blood was separated into plasma and erythrocytes, and the drug concentration in the plasma was measured. The pharmacokinetic parameters were obtained from the FLRX concentration, using a personal computer program of PHAconet-one (System Wave Co. Ltd.) for non-linear least-squares regression. The maximum plasma concentration (C_{max}) and its corresponding time (T_{max}), the absorption rate constant (K_a), the elimination half-life ($T_{1/2}$) and the area under the plasma concentration–time curve ($AUC_{0\rightarrow\infty}$) were estimated by the computer program.

3. Results

3.1. Chromatography

As shown in Fig. 1, the retention times of FLRX and the I.S. were 6.6 and 13.4 min, respectively. No other peaks corresponding to these retention times were noted on the chromatogram of drug-free plasma, indicating that no interfering endogenous substances were present.

3.2. Calibration curve

The standard curve was prepared by analyzing a FLRX plasma solution at a concentration of 125, 250, 500, 1000, 2000, 4000 and 8000 ng/ml. Between the concentration of FLRX in blood and peak-area ratio of FLRX to I.S., an excellent linear

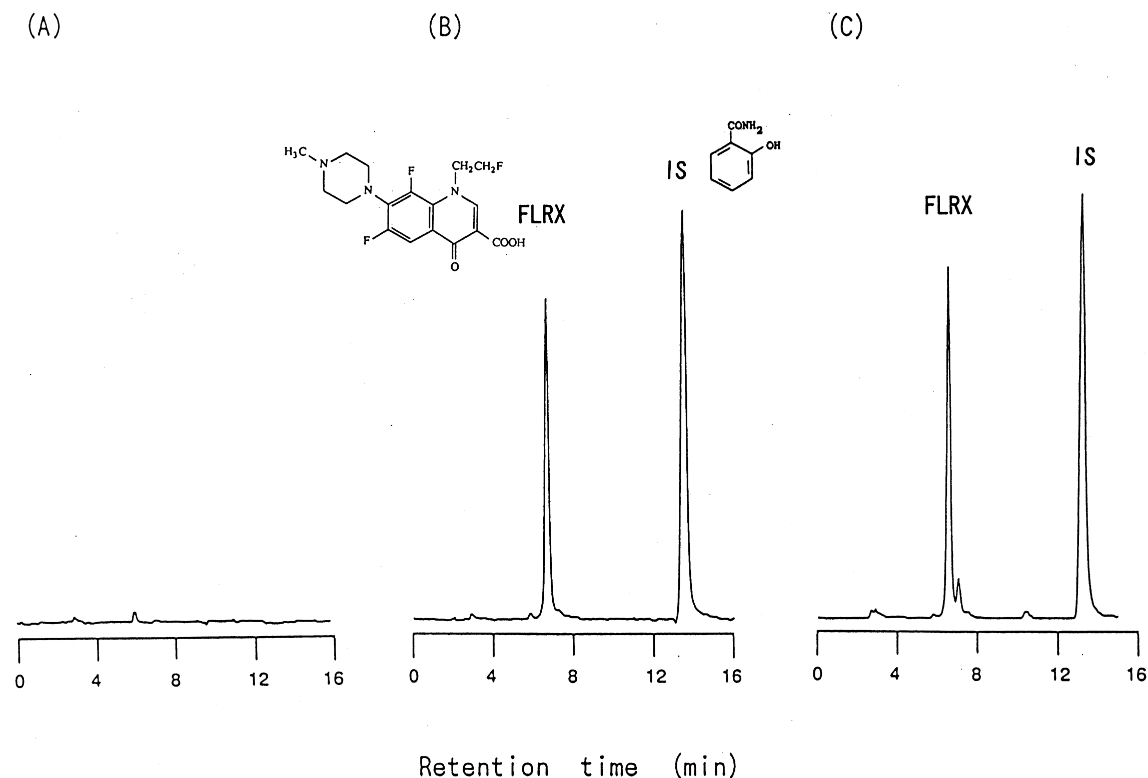

Fig. 1. Typical chromatograms for FLRX and internal standard (I.S.). (A) Drug-free plasma; (B) spiked plasma (FLRX 1.0 μg/ml, I.S. 30 μg/ml); (C) rat plasma at 60 min following oral administration of FLRX at a dose of 10 mg/kg body weight. The rat plasma concentration of FLRX was 1.1 μg/ml, calculated from peak–area ratio of FLRX to I.S.

Table 1
Recovery test and within-day assay in method proposed for determination of FLRX

Added FLRX on plasma (A) (μg/ml)	n	Measured (B) (μg/ml)	Recovery (A/B) (%)	Within-day assay C.V. (%)
0.25	3	0.27±0.01	106.8	2.60
0.50	3	0.52±0.01	104.5	2.21
1.00	4	0.99±0.01	99.0	0.50
2.00	4	1.94±0.06	97.2	3.22
4.00	3	3.97±0.07	99.3	1.65
8.00	3	8.13±0.05	101.7	0.96

Each value is the mean±S.D.

correlation was noted in the range 125–8000 ng/ml. The coefficient of correlation (r) was 0.999 and the regression equation was $y=0.392x-0.009$. The limit of detection at a signal-to-noise ratio of 3 was 58 ng/ml.

3.3. Recovery rate and precision

The rates of recovery calculated by addition of 0.25, 0.50, 1.00, 2.00, 4.00 and 8.0 μg/ml to plasma samples of a non-drugged rat averaged 1001.4% (97.2–106.8%). To test the precision of the assay of FLRX plasma levels, the present method was used to perform a within-day assay and a between-day assay. The coefficients of variation (C.V.) in the FLRX added plasma sample were 0.50–3.22% in the within-day assay (Table 1). The C.V. in the FLRX added plasma sample was 2.87% (1.06±0.03 μg/ml) in the between-day assay (7 days).

3.4. Concentration–time profile of FLRX

Concentration–time profile for plasma FLRX following administration of doses of 10 and 20 mg/kg are shown in Fig. 2. The concentration of FLRX in the plasma increased in a dose-dependent fashion. For each administration group, the values of K_a, C_{max}, $T_{1/2}$ and AUC are shown in Table 2. Thus, the pharmacokinetics of FLRX in small animals can be studied by this method.

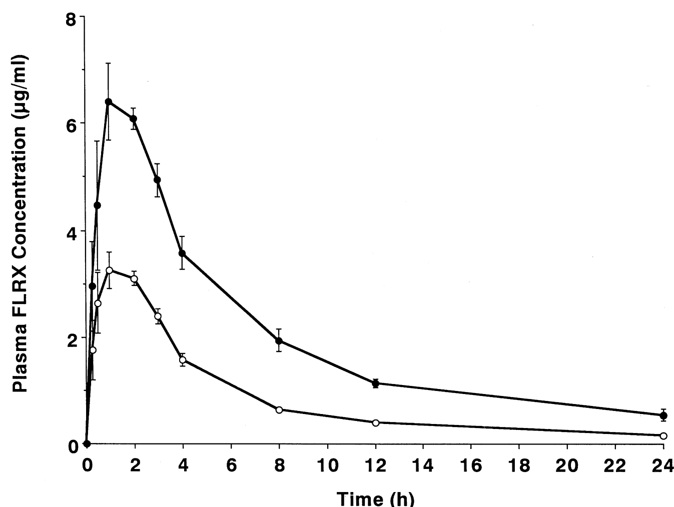

Fig. 2. Plasma FLRX concentrations after oral administration in rats. Each point represents the mean value for five rats, and each vertical line the standard error of the mean. (○) 10 mg/kg ; (●) 20 mg/kg.

Table 2
Pharmacokinetic parameters of FLRX after oral administration

FLRX dose (mg/kg)	K_a (h^{-1})	T_{max} (h)	C_{max} (μg/ml)	$T_{1/2}$ (h)	$AUC_{0\to\infty}$ (μg·h/ml)
10	2.156±0.842	1.29±0.24	3.47±1.29	2.11±0.17	16.12±0.54
20	1.985±0.600	1.50±0.31	6.52±0.45	3.07±0.46	39.71±3.03

Each value is the mean±S.E.M.

4. Discussion

Preliminary reports indicated that FLRX has broad and potent antibacterial activity against Gram-negative and Gram-positive bacteria and that it is well absorbed after oral administration in various experimental animals [1,2]. In order to evaluate the therapeutic effect, the dose of FLRX must be established and the interactions among these drug concentrations in the plasma must be studied pharmacokinetically. The disappearance of side effects is dose dependent and is related to the value of trough and hence the plasma FLRX must be monitored. Thus we developed a rapid and simple method for the determination of FLRX in rat plasma. This method showed a favorable recovery (97.2–106.8%) after pretreatment with Bond Elut, as well as good reproducibility (C.V. 0.50–3.22%). Also the plasma concentration could be determined using a small volume of plasma (20 μl), such as that obtained from a rat tail vein. In addition, the assay was rapid and easy to perform. The plasma concentrations in the cervical vein and tail vein showed a good correlation (r=0.957). By sampling from rat tail veins the time-course of plasma FLRX following oral administration of 10 mg/kg could be observed. This method is useful in basic studies of plasma FLRX in small animals. It is a rapid and simple way for microdetermination in plasma and obtaining the plasma FLRX concentration–time data from a single animal, together with further pharmacokinetic parameters, such as K_a, T_{max}, C_{max} and $T_{1/2}$. The feasibility of observing plasma FLRX dynamics in a chronological sequencer presents an advantage. For instance, the relationship between the blood concentration and the pharmacological activity can be studied, and the effect of any other drugs used concurrently, or other influencing factors can be taken into account.

Clinical doses of FLRX produce a plasma concentration of 1.6–15.6 μg/ml at the C_{max} [9]. The present method can be used to detect a concentration of 58 ng/ml which could be specifically measured in less than 22 min after simple extraction process. This method may be useful in clinical pharmacology for detecting the efficacy and side effects of FLRX.

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