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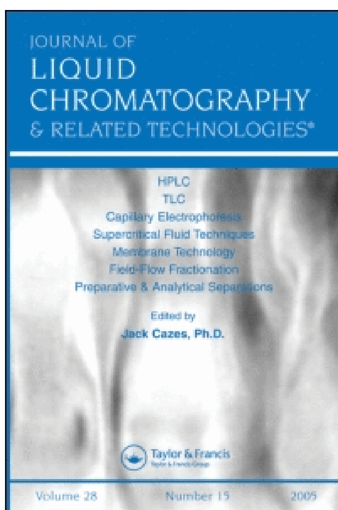

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DETERMINATION OF SPARFLOXACIN IN PLASMA BY DIRECT INJECTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH COLUMN SWITCHING

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ABSTRACT

High performance liquid chromatography (HPLC) assay was developed for the determination of sparfloxacin in plasma. The plasma samples were directly introduced onto a HPLC column after filtering through a Molcut II® membrane filter, which removes high molecular proteins. The sparfloxacin in filtrate was separated from interfering substances and concentrated on a pre-column using an ODS stationary phase and then introduced to an analytical column with an ODS stationary phase by column switching. Sparfloxacin and 4'-oxo-enoxacin, as an internal standard, were detected by ultraviolet absorbance at 300 nm. Determination of sparfloxacin was possible over the concentration range 50-2000 ng/mL; the limit of detection was 20 ng/mL. The recovery of sparfloxacin added to plasma was 97.5-100.3 % with a relative standard deviation of less than 2.2 %. This method is applicable to pharmacokinetic studies.

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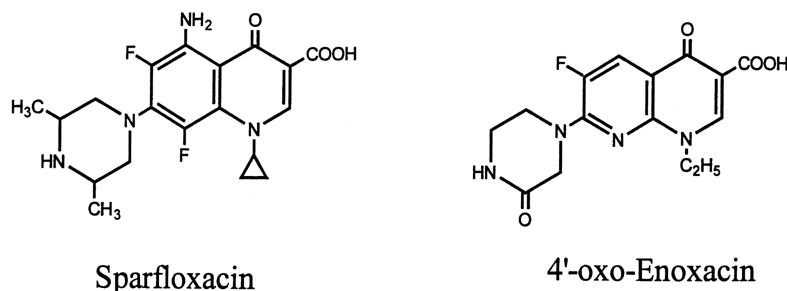

Figure 1. Chemical structures of sparfloxacin and 4'-oxo-enoxacin.

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INTRODUCTION

Sparfloxacin, 5–amino–1– cyclopropyl -6, 8 –difluoro -1, 4- dihydro- 7-(cis-3, 5- dimethyl-piperazinyl)-4-oxoquinoline-3-carboxylic acid (Fig.1), is a new fluoroquinolone that has a broad antibacterial spectrum covering gram-positive and gram-negative bacteria in vitro and in vivo.[1] Previous new quinolones have uncertain antibacterial activity for Mycobacterium, Mycoplasma, and Chlamydia, however, sparfloxacin has reliable antibacterial potency against these bacterias.[1] Previous papers have described HPLC methods for the determination of sparfloxacin level in plasma or urine.[2,3] K.Borner and E.Borner described a protein precipitation method using acetonitrile.[4] The former method is tedious and time consuming, while the latter causes damage to columns due to the accumulation of endogenous substances.

A direct injection method using column switching without extraction has been described in our previous papers.[5-7] This method is very useful for the determination of low recovery in an extraction method and unstable compounds in biological samples. This method is simple and rapid. In the present paper, we describe a simple and sensitive HPLC method for determination of the sparfloxacin in human plasma by column switching techniques. This method was used for the analysis of plasma samples obtained from volunteers.

EXPERIMENTAL

Materials

Sparfloxacin and 4'-oxo-enoxacin (Fig.1) were kindly donated by Dainippon Pharmaceutical Co. (Tokyo, Japan). A Molcut II® membrane filter

was obtained from Millipore Co. (Bedford, MA, USA). All other solvents were used of HPLC grade (Wako Pure Chemicals Industries Ltd. Tokyo, Japan). All other reagents and chemicals were purchased from Wako Pure Chemicals Industries or Nakarai Tesque (Kyoto, Japan). All reagents used were analytical grade.

Apparatus

The apparatus used for HPLC was a Jasco Model PU-880 chromatography pump (Jasco Co.Ltd. Tokyo, Japan) equipped with a Jasco Uvidec 880 ultraviolet detector (Jasco). The detection wavelength was set at 300 nm. Test samples were injected using a Model 7125 injector (Rheodyne, Cotati, CA, USA) with an effective volume of 500µL. HPLC was carried out using Develosil ODS-5 (50 x 4.6 mm, I.D.) as a precolumn, and separation of sparfloxacin and 4'-oxo-enoxacin as an internal standard was performed on a Develosil ODS-5 column (150 x 4.6 mm, I.D.) (Nomura Chemical Co., Seto, Japan) at 1.0 mL/min of flow rate in ambient temperature. The mobile phase consisted of 0.5 % sodium acetate (pH 2.5) : acetonitrile (80:20, v/v). Before mixing, the pH of the mobile phase was adjusted with 50% phosphoric acid and then degassed ultrasonically.

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Determination of Sparfloxacin in Human Plasma

4'-Oxo-enoxacin (2000 ng) in methanol (10µL) was added to the plasma samples (500µL) as an internal standard and then 100µL of ethanol was added. The mixture was filtered through a Molcut II® membrane filter for deprotenization. The filtrate (60µL) was then loaded onto the precolumn for the elimination of interfering substances in plasma and the concentration of sparfloxacin and 4'-oxo-enoxacin on the precolumn. After washing for 1.8 min, sparfloxacin and 4'-oxo-enoxacin were eluted from the precolumn and then led to the analytical column by column-switching technique using 0.5 % sodium acetate (pH 2.5)-acetonitrile (80:20, v/v) as a single mobile phase. The procedure was carried out on-line with one pump (Fig. 2).

Calibration Graphs

Known amounts of sparfloxacin in the range 50-2000 ng/mL were added to blank plasma samples. These plasma samples were treated according to the method described above. The peak-height ratios of sparfloxacin to 4'-oxo-enoxacin were measured and plotted against the concentration of sparfloxacin in plasma.

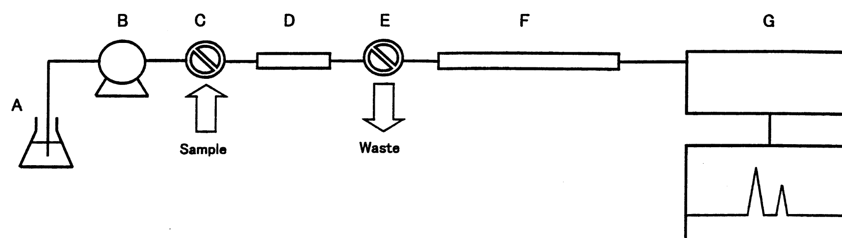

Figure 2. Schematic diagram of the column switching HPLC system. A: carrier solution; B: LC pump; C: injector; D: precolumn; E: 3-way valve; F: analytical column; G: UV detector.

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Recovery Experiments

Plasma was spiked by adding a known amount of sparfloxacin (200, 500, 1000 and 2000 ng/mL) to drug free plasma to obtain a total volume of 0.5 mL. These samples were treated by the above described method. Control samples were prepared by adding a known amount of sparfloxacin to 0.5 mL water. These control samples were treated by Molcut II® according to the previous described method. An external standard instead of the internal standard was added to all of the samples after Molcut II® treatment. Recoveries were determined by comparison between plasma samples and water samples.

Method Validation

The accuracy of the method was determined by injection of the sparfloxacin calibration samples and then the four different concentration quality-control samples after Molcut II® treatment on six separate days. All calibration curves were required to have a correlation value of at least 0.99. The accuracy was calculated as a percentage of the nominal concentration: Accuracy = (Conc.obs/Conc.nominal) x 100%. The same data used in the accuracy determinations were used for the calculation of the between-run percentage relative standard deviation [%R.S.D.: RSD = (SD/mean) x 100 %].

The within-run % R.S.D. was derived from analysis of six quality control samples at each concentration with injection on the same day. The detection limit of the HPLC assay after extraction was estimated from the drug quantity in plasma which corresponded to five times the baseline noise. The lower limit of quantitation was defined as the quantity of the sample after preparation, which was quantified with deviation and precision less than 10%.

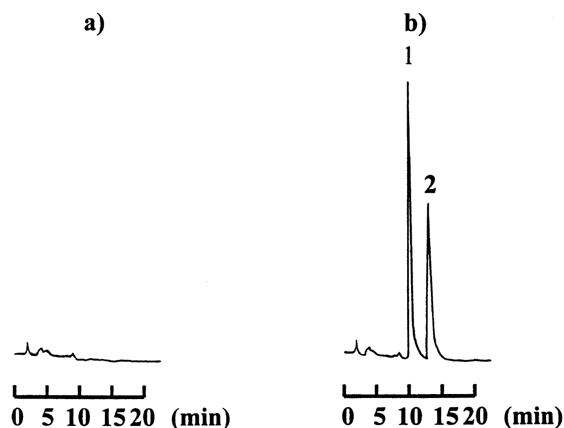

Figure 3. Chromatograms of a) a plasma blank, b) a plasma blank with sparfloxacin and 4'-oxo-enoxacin. Peak 1: sparfloxacin; Peak 2: 4'-oxo-enoxacin.

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Drug Administration and Sampling

Sparfloxacin tablets (200 mg)(two tablets of Spara® (100 mg) brand of sparfloxacin, Dainippon Pharmaceutical Co.) were orally administrated to six healthy volunteers. Blood samples (5 mL) were collected by venipuncture at 1, 2, 3, 4, 6, 8, 10, and 24 hr after administration. Plasma samples were separated by centrifugation at 1900 x g for 15 min and stored at -40°C until analysis.

RESULTS

The effects of pH and ratio of organic solvent of the mobile phase on the retention factors (κ) of sparfloxacin were studied using six kinds of stationary phase (ODS, C_8, C_1, CN, Ph, tert-But)(date was not shown). The κ values of sparfloxacin increased with higher pH when 0.5 % sodium acetate-acetonitrile (80:20, v/v) was used. Sparfloxacin was retained more strongly on octadecyl stationary phase. The best resolution and most suitable analytical time was obtained when 4'-oxo-enoxacin was chosen as the internal standard, which was therefore, used as the internal standard.

The separation between endogenous substances and sparfloxacin or 4'-oxo-enoxacin was achieved using octadecyl stationary phase on the precolumn and analytical column with 0.5 % sodium acetate (pH 2.5)-acetonitrile (80:20, v/v). Figure 3 shows representative chromatograms for a blank plasma samples and a plasma sample obtained from a healthy volunteer administrated with

Table 1

Recovery of Sparfloxacin Added to Human Plasma

Added (ng/mL)	Found (ng/mL) (Mean ± S.D.)	Recovery (%) (Mean ± S.D.)	RSD (%)
200	199.3 ± 3.3	99.7 ± 1.6	1.6
500	501.8 ± 7.0	100.3 ± 1.4	1.4
1000	973.2 ± 18.4	97.3 ± 1.8	1.8
2000	1949.4 ± 44.0	97.5 ± 2.2	2.2

sparfloxacin tablet (200 mg). No significant interference peaks derived from plasma were seen on the chromatogram. Calibration graphs for sparfloxacin in human plasma were linear in the range of 50-2000 ng/mL (r = 0.99). The limit of detection for sparfloxacin was 20 ng/mL (signal-to-noise ratio = 5). The results of recovery studies are shown in Table 1.

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The recovery of sparfloxacin was determined by adding the four known levels of 200, 500, 1000, and 2000 ng/mL to blank plasma. The recovery values for sparfloxacin were 97.3-100.3 % in plasma. Relative standard deviations were less than 2.2 %. The change in concentration of sparfloxacin over time in plasma samples from six healthy volunteers who received 200 mg sparfloxacin orally was determined using the proposed method (Fig. 4). Pharmacokinetic parameters of six healthy volunteers were calculated and shown in Table 2.

DISCUSSION

Initially, our efforts were directed toward developing an efficient chromatographic system for the direct injecting determination of plasma sparfloxacin using column-switching techniques. In our previous paper,[7] we studied the effects of pH of the mobile phase on the retention factors (κ) of several quinolone derivatives using six kinds of stationary phase.

In this study, we also examined the characterization of sparfloxacin and 4'-oxo-enoxacin on six kinds of stationary phase columns. Octadecyl stationary phase showed strongest retention power, good separation and suitable analytical time on the analytical column. On the other hand, best purification efficiency of sparfloxacin and 4'-oxo-enoxacin from interference substances in plasma on the precolumn was obtained with octadecyl stationary phase.

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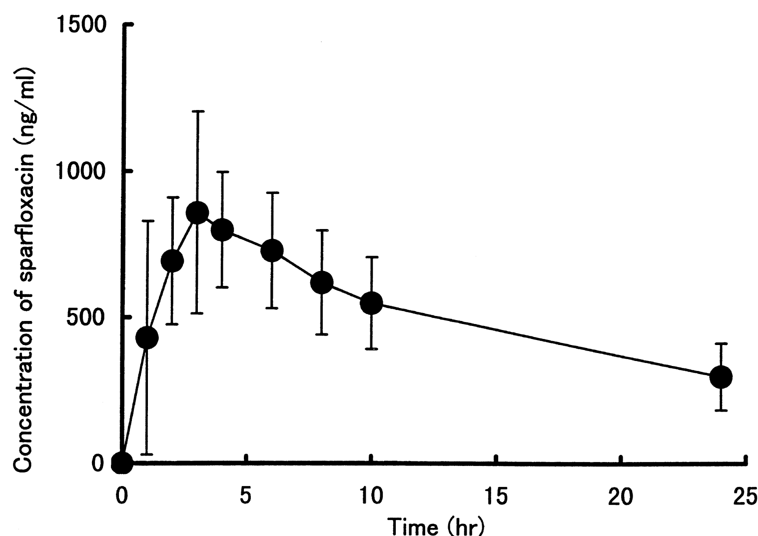

Figure 4. Time course of sparfloxacin concentration in plasma after administration of oral dose of 200mg for six healthy volunteers.

Table 2

Pharmacokinetic Parameter of Sparfloxacin in Healthy Volunteers*

Sparfloxacin Dose (mg)	Cmax (ng/mL) (Mean ± S.D.)	Tmax (hr)	Ke (hr^{-1}) (Mean ± S.D.)	T 1/2 (hr) (Mean ± S.D.)	AUC (0-∞) (ng•hr/mL) (Mean ± S.D.)
200	857.9 ± 245.4	3.0	0.053 ± 0.013	13.7 ± 3.6	19744.3 ± 7638.4

* (n = 6).

In our previous paper,[7] we described that the different interactive combination of stationary phase between a precolumn and an analytical column obtained good separation of quinolone derivatives from endogenous interfering substances by column-switching techniques with series-connection column system. However, we had good separation of sparfloxacin and 4'-oxo-enoxacin as an internal standard, which was free of interfering endogenous substances by using a combination system of octadecyl precolumn, and octadecyl analytical column. This fact is explained by the observation that the octadecyl phase had a strong retaining power for quinolone derivatives, which were retained on stationary phase for obviously longer periods of time than interfering substances

from plasma. The separation of sparfloxacin has been achieved by octadecyl column using gradient mobile phase[2,3] and cation-exchange column[4] as described in previous papers. However, gradient mobile phase is tedious and requires time for column recycle washing. Also, Ion-exchange chromatography is required for the adjustment of cation concentration, pH in mobile phase, and pH of sample. Using our present method, we were able to avoid these problems. In addition, extraction procedures required in previous methods[2-4] were tedious.

In our present method, we could measure sparfloxacin in plasma using direct injection method without extraction procedure. This method saves time on the assay procedure, which can be carried out as a simple procedure. No significant interfering peaks derived from biological substances were seen on the chromatogram (Fig.3). The results of recovery studies show that the proposed method is both accurate and precise. Especially, recovery rate reached to about 100 %, and the relative standard deviation obtained was less than by any previous study has reported.[2-4] Based on the results of this data, we considered our present method is simple to perform with a high degree of confidence in the quality and accuracy of results. Therefore, we recommend this method for use in future pharmacokinetic studies and as a medication assay procedure for patients receiving sparfloxacin treatment.

The method described was used to study the pharmacokinetics of sparfloxacin at oral dose of 200 mg in healthy volunteers (Fig.4). In the previous paper, Sakashita et al.[8] described the pharmacokinetics of sparfloxacin. The pharmacokinetic parameters obtained in the present study (Table 2) are roughly similar to those of the previous paper.[8] Plasma concentration of sparfloxacin was affected by ingestion of food.[8] Therefore, we consider the results of our present work as confirming the findings of this previous study, as the results are nearly identical.[8] From the present results, it was shown that the proposed method for the determination of sparfloxacin could be applied to pharmacokinetic studies in sparfloxacin treated volunteers and patients. It is well known that fluoroquinolones are involved in a number of clinically important drug-drug interaction.[9] Plasma concentration of sparfloxacin was decreased by co-administration with aluminum hydroxide.[10] Therefore, further pharmacokinetic studies of drug-drug interaction will be carried out in these laboratories, and details will be reported elsewhere.

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