Journal of Chromatography B, 878 (2010) 1555–1561

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous measurement of pazufloxacin, ciprofloxacin, and levofloxacin in human serum by high-performance liquid chromatography with fluorescence detection

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ARTICLE INFO

Article history:
Received 7 December 2009
Accepted 11 April 2010
Available online 1 May 2010

Keywords:
Pazufloxacin
Ciprofloxacin
Levofloxacin
HPLC
Validation
Serum

ABSTRACT

In this study, three fluoroquinolones, pazufloxacin, ciprofloxacin and levofloxacin, were simultaneously determined in spiked human serum by high-performance liquid chromatography (HPLC) method with fluorescence detection. Chromatography was performed using a C_8 column with an isocratic mobile phase consisting of 1% triethylamine (pH 3.0)/acetonitrile (86/14, v/v). Protein precipitation was conducted using perchloric acid and methanol. The calibration curves for the three fluoroquinolones were linear over concentrations ranging from 0.1 to 20.0 μg/mL. The within-day and between-day coefficients of variation obtained from three fluoroquinolones were less than 7%, and relative errors ranged from −1.6% to 9.3%. Mean recoveries of pazufloxacin, ciprofloxacin, and levofloxacin from spiked human serum were 97%, 88%, and 90%, respectively. The proposed method proved to be simple and reliable for the determination of three fluoroquinolones.

1. Introduction

Pazufloxacin, ciprofloxacin, and levofloxacin are fluoroquinolone antibiotics that exhibit strong activities against both gram-positive and gram-negative bacteria. The mechanism of their activity is based on the inhibition of bacterial DNA gyrase [1,2]. The structures of these three fluoroquinolones are shown in Fig. 1. Although fluoroquinolones are generally well tolerated [3,4], they cause severe adverse effects such as convulsion-inducing activity in rare cases [5]. The antibiotic activity, efficacy, and tolerability of ciprofloxacin and levofloxacin have been evaluated previously [6–10].

Pazufloxacin was developed as a new injectable fluoroquinolone in Japan [11,12]. This agent has relatively potent *in vitro* antibacterial activity in comparison with the antibacterial activities of similar quinolones [12,13]. Previous studies reported that the adverse effects of pazufloxacin, such as drug-induced convulsion and hypotension are less than those of other conventional injectable fluoroquinolones [11,14].

Fluoroquinolones generally show concentration-dependent bacteria-killing activity that depends on the ratio of maximum drug concentrations (C_{max}) to minimum inhibitory concentration (MIC) [15]. In addition, it has been reported that the ratio of the 24 h area under the concentration–time curve (AUC_{24}) of fluoroquinolones to MIC is the most important predictor of clinical cure [16–18]. These findings suggest that monitoring the concentration of fluoroquinolones in serum is useful to make effective drug dosage regimens and to prevent bacterial resistance. In addition, simple analytical condition and sample preparation methods are useful in routine clinical practice.

Many researchers have determined fluoroquinolone concentrations in serum or plasma using high-performance liquid chromatography (HPLC) with a UV [19–23] or fluorescence detector [24–26]. Several researchers have developed methods for separation and simultaneous quantification of two or more fluoroquinolones, including ciprofloxacin, in human serum or plasma [27–29]. Neckel et al. [30] developed a method for simultaneous quantification of ciprofloxacin and levofloxacin using a simple sample preparation method. Hasegawa et al. [23] developed a method for the determination of pazufloxacin in rat plasma. However, this method involved time-consuming sample preparation.

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doi:10.1016/j.jchromb.2010.04.012

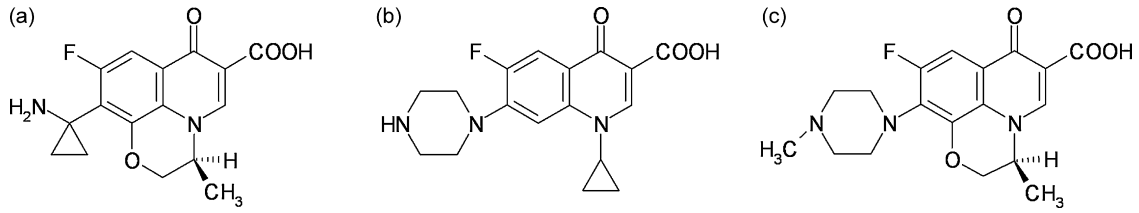

Fig. 1. Chemical structures of (a) pazufloxacin, (b) ciprofloxacin, and (c) levofloxacin.

There is no published report for simultaneously measuring pazufloxacin, ciprofloxacin, and levofloxacin in human serum until now.

The present study aimed to develop a simple and reliable HPLC method for simultaneous measurement of pazufloxacin, ciprofloxacin, and levofloxacin concentrations in spiked human serum.

The proposed method was carried out without changing the analytical conditions and utilized a simple sample preparation method. In future, this method will help in clinical study and routine analysis.

2. Experimental

2.1. Chemicals and human drug-free serum control

Pazufloxacin was donated by Mitsubishi Tanabe Pharma Factory, Ltd. (Osaka, Japan). Ciprofloxacin and levofloxacin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and acetonitrile (both HPLC-grade) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Hydrochloric acid, perchloric acid (60%, w/v), triethylamine, and phosphoric acid were purchased from Wako Pure Chemical Industries, Ltd. Lyphochek® drug-free serum, a commercial human drug-free serum control, was purchased from Bio-Rad Laboratories, Inc. (California, USA).

2.2. Apparatus

Details of the HPLC apparatus are as follows: pump, IP-7500 (Lab-Quatec Co., Tokyo, Japan); degasser, DG-2080-53 (Jasco Co., Tokyo, Japan); autosampler, Autosampler Model 09 (System Instruments Co., Ltd., Tokyo, Japan); fluorescence detector, FP-2020 Plus (Jasco Co.); integrator and recorder, Chromatocorder 21 (System Instruments Co.).

2.3. Chromatography

Chromatographic separations were carried out at room temperature on an Inertsil® C_8-3 column (250 mm × 4.6 mm i.d., particle size 5 μm; GL Sciences, Tokyo, Japan) with an Inertsil® C_8-3 guard column (10 mm × 4.0 mm i.d., particle size 5 μm; GL Sciences). The mobile phase was isocratic, consisting of 1% triethylamine (pH 3.0)/acetonitrile (86/14, v/v). The pH of 1% triethylamine was adjusted to 3.0 using phosphoric acid. The flow rate was 1.0 mL/min. The fluorescence detection wavelengths were set to 300 nm (excitation) and 450 nm (emission). The run time was 18 min per sample.

2.4. Standard solutions

Stock standard solutions of pazufloxacin and levofloxacin were prepared in water at 2.0 mg/mL, and the stock standard solution of ciprofloxacin was prepared in 0.1 M HCl at 2.0 mg/mL. Working standard solutions of pazufloxacin (800 μg/mL) and levofloxacin (800 μg/mL) were prepared by diluting their respective stock standard solutions with water. The working standard solution of ciprofloxacin (400 μg/mL) was prepared by diluting its stock standard solution with 0.1 M HCl. These stock and working standard solutions were stored at −20 °C. The working standard solutions of pazufloxacin, levofloxacin, and ciprofloxacin were mixed at 1/1/2 (v/v/v). This working standard mixture was freshly prepared daily. The working standard mixture was diluted with 1% triethylamine (pH 3.0) to the required concentrations and used to prepare calibration standards and quality control (QC) samples.

2.5. Calibration standards and QC samples

In this study, Lyphochek® drug-free serum (Bio-Rad Laboratories) was used as the drug-free human serum. The calibration standards and QC samples were prepared by adding 20 μL of the working standard mixture to an aliquot of 180 μL drug-free human serum.

2.6. Sample preparation

One hundred microliters of 6.0% (w/v) perchloric acid was added to 200 μL of calibration standards or QC samples, and then vortexed for approximately 30 s. Subsequently, 100 μL of methanol was added to the above mixture, then vortexed for approximately 30 s. The mixture that was obtained after deproteinization using perchloric acid and methanol was centrifuged at 15,600 × *g* for 12 min at room temperature. After centrifugation, 20 μL of the supernatant was injected into the HPLC apparatus.

2.7. Validation

Validation was carried out according to the Guidance for Industry: Bioanalytical Method Validation published by United States Food and Drug Administration (FDA) [31]. The HPLC method was validated for linearity, accuracy and precision, recovery, and stability. Precision and accuracy were determined in terms of the coefficient of variation (CV%) and relative error (RE%), respectively. FDA guidance is described as follows: the precision should not exceed 15% except for the lower limit of quantification (LLOQ), where it should not exceed 20%. The accuracy should not exceed ±15%, except for the LLOQ, where it should not exceed ±20%.

In this study, CVs of recovery and stability were set as ≤15%. The correlation coefficient (r^2) of the calibration curve was set above 0.99.

2.7.1. Linearity of calibration curves

Calibration curves of the three fluoroquinolones were constructed using eight calibration standards over a calibration range of 0.1–20.0 μg/mL (0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 15.0, and 20.0 μg/mL). Five calibration curves constructed on five separate days were analyzed to evaluate the linearity of each calibration curve.

Peak areas of the fluoroquinolones were plotted against the corresponding concentrations. The linear equation of the calibration curves was determined by the weighted (1/concentration^2) least-squares regression method. The calibration curves were described by the following linear equation: $Y = aX + b$, where Y is the ana-

Table 1
Slope, intercept, and correlation coefficient (r^2) of calibration curves on five separate days ($n = 5$) (mean (SD)).

	Slope	Intercept	r^2
Pazufloxacin	1297556 (24837)	−9231 (3660)	0.9996 (0.0001)
Levofloxacin	2861896 (114641)	−24433 (4671)	0.9997 (0.0001)
Ciprofloxacin	3504821 (68206)	−17035 (11300)	0.9995 (0.0001)

lyte area and X is the concentration (μg/mL). The linearity of each calibration curve was evaluated from the slope, intercept, and correlation coefficient (r^2) of the curve. Linearity was also confirmed from back-calculated concentrations of the calibration standards.

2.7.2. Precision, accuracy, and recovery

Four different concentrations (0.1, 0.2, 8.0, and 16.0 μg/mL) of QC samples were prepared. The within-day precision and accuracy of the proposed method were evaluated by analyzing five extracts of each QC sample on the same day. To determine the between-day precision and accuracy, the QC samples were analyzed in three replicates for seven days. The recoveries of fluoroquinolones were determined by comparing the analyte peak areas of extracted QC samples with those of reference standard solutions prepared at the same concentration. The reference standard solutions were prepared by adding 20 μL of the working standard mixture to 180 μL of water and then adding 100 μL of 6.0% (w/v) perchloric acid and 100 μL of methanol.

2.7.3. Stability evaluation

The stabilities of stock standard solutions were investigated under two conditions: after storage at room temperature for 24 h and at −20 °C for 30 days. The stabilities were measured by comparison between the peak area of stock standard solutions and that of freshly prepared solution. Five samples were used in this study.

The stabilities of the fluoroquinolones in drug-free human serum were investigated experimentally under three conditions: after three freeze-thaw cycles, after storage at room temperature for 24 h, and at −20 °C for three months. The stabilities of the fluoroquinolones in drug-free human serum were investigated by using the QC samples analyzed in triplicates. These test samples were compared to the freshly prepared samples ($n = 3$).

3. Results

3.1. Separation and limits of quantitation

Pazufloxacin, levofloxacin, and ciprofloxacin were successfully separated by this HPLC method (Fig. 2a).

No interfering peaks were observed in the drug-free human serum after deproteination (Fig. 2b and c). The quantitation limits for the three fluoroquinolones were defined as the lowest drug concentrations on the calibration curves (0.1 μg/mL). For all three fluoroquinolones, the precision (CV) and accuracy (RE) at a concentration of 0.1 μg/mL were ≤20% and ≤±20%, respectively (Table 2).

3.2. Vaidation

3.2.1. Linearity of calibration curves

The linearity of the calibration curves for all three fluoroquinolones in drug-free human serum over the 0.1–20.0 μg/mL range was evaluated (Table 1). The correlation coefficients (r^2) (mean (SD)) for the calibration curves for pazufloxacin, levofloxacin, and ciprofloxacin were 0.9996 (0.0001), 0.9997 (0.0001), and 0.9995 (0.0001), respectively. The equations of the calibration curves were as follows: pazufloxacin: $Y = 1{,}297{,}556X - 9231$, levofloxacin: $Y = 2{,}861{,}896X - 24{,}433$,

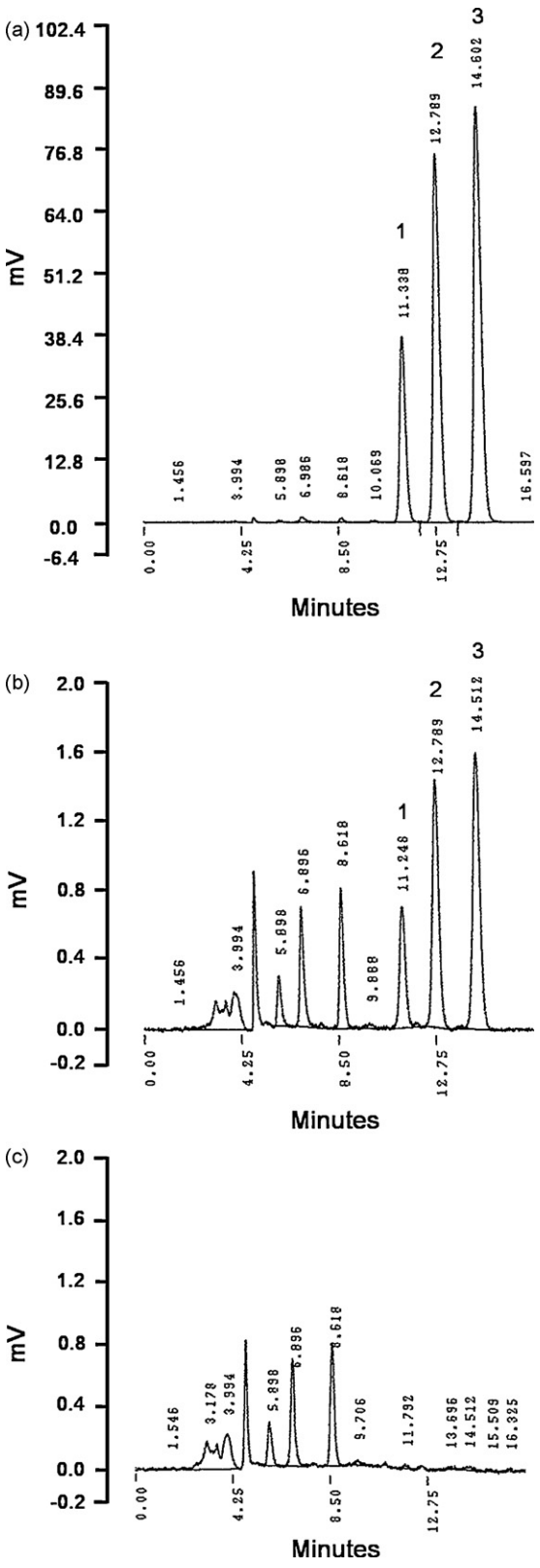

Fig. 2. Representative chromatograms obtained from drug-free human serum spiked with (a) 5.0 μg/mL or (b) 0.1 μg/mL fluoroquinolones and (c) drug-free human serum. 1, pazufloxacin; 2, levofloxacin; 3, ciprofloxacin.

and ciprofloxacin: $Y = 3{,}504{,}821X - 17{,}035$. The back-calculated concentrations of the calibration standards are listed in Table 2. The range of precision (CV%) of back-calculated concentrations of pazufloxacin, levofloxacin, and ciprofloxacin was 0.5–2.7%, 0.5–1.6%, and 0.5–1.8%, respectively. The accuracy (RE%) of back-calculated concentrations of pazufloxacin, levofloxacin, and ciprofloxacin ranged from −2.6% to 3.1%, −2.6% to 3.3%, and −3.0% to 3.8%, respectively. The precision and accuracy of the back-calculated concentrations were acceptable.

3.2.2. Precision, accuracy, and recovery

The within-day and between-day precisions and accuracies were evaluated using QC samples (Table 3). For all three fluoroquinolones, the within-day and between-day precisions were less than 7% at all four concentration levels. Furthermore, the within-day and between-day accuracies for all three fluoroquinolones ranged from −1.6% to 9.3%.

The range of mean recoveries of pazufloxacin, levofloxacin, and ciprofloxacin by the proposed method was 96–99%, 86–91%, and 85–90%, respectively (Table 4). These results indicated that the proposed method was precise and accurate.

3.2.3. Stability

The stabilities of stock standard solutions, stored at room temperature for 24 h and at −20 °C for 30 days, were tested using five replicates. The peak area ratios of stock standard solutions stored at room temperature for 24 h to freshly prepared solutions (mean (CV)) were as follows: pazufloxacin, 101.9% (1.1%); levofloxacin, 100.9% (1.6%); and ciprofloxacin, 101.2% (1.1%). In addition, the peak area ratios of stock standard solutions stored at −20 °C for 30 days to freshly prepared solutions (mean (CV)) were as follows: pazufloxacin, 100.6% (0.9%); levofloxacin, 100.4% (0.9%); and

Table 2
Validation of five calibration curves for pazufloxacin, levofloxacin, and ciprofloxacin constructed on five separate days.

Nominal concentration (μg/mL)	Calculated concentration (Mean (SD)) (μg/mL)	RE (%)	CV (%)
Pazufloxacin			
0.1	0.102 (0.001)	1.8	1.1
0.2	0.196 (0.005)	−2.2	2.7
0.5	0.487 (0.008)	−2.6	1.6
1.0	0.978 (0.019)	−2.2	1.9
5.0	4.985 (0.035)	−0.3	0.7
10.0	10.087 (0.055)	0.9	0.5
15.0	15.212(0.090)	1.4	0.6
20.0	20.629(0.161)	3.1	0.8
Levofloxacin			
0.1	0.102 (0.001)	1.6	0.5
0.2	0.196 (0.002)	−1.8	1.2
0.5	0.488 (0.004)	−2.5	0.7
1.0	0.974 (0.015)	−2.6	1.6
5.0	4.991 (0.031)	−0.2	0.6
10.0	10.079 (0.060)	0.8	0.6
15.0	15.210 (0.069)	1.4	0.5
20.0	20.651 (0.165)	3.3	0.8
Ciprofloxacin			
0.1	0.102 (0.001)	1.8	0.8
0.2	0.196 (0.004)	−2.0	1.8
0.5	0.486 (0.003)	−2.8	0.7
1.0	0.970 (0.013)	−3.0	1.3
5.0	4.981 (0.038)	−0.4	0.8
10.0	10.088 (0.046)	0.9	0.5
15.0	15.255 (0.079)	1.7	0.5
20.0	20.760 (0.171)	3.8	0.8

RE: relative error, CV: coefficient of variation.

Table 3
Between-day and within-day precisions and accuracies for pazufloxacin, levofloxacin, and ciprofloxacin.

Nominal concentraion (μg/mL)	Within-day assays ($n = 5$)			Between-day assays ($n = 21$)		
	Calculated concentration (Mean (SD)) (μg/mL)	RE (%)	CV (%)	Calculated concentration (Mean (SD)) (μg/mL)	RE (%)	CV (%)
Pazufloxacin						
0.1	0.101 (0.004)	1.0	3.5	0.102 (0.005)	2.5	4.6
0.2	0.200 (0.005)	0.1	2.5	0.203 (0.007)	1.4	3.3
8.0	8.745 (0.259)	9.3	3.0	8.052 (0.324)	0.6	4.0
16.0	16.076 (0.301)	0.5	1.9	16.051 (0.250)	0.3	1.6
Levofloxacin						
0.1	0.103 (0.001)	2.9	1.4	0.104 (0.004)	4.4	4.1
0.2	0.200 (0.003)	0.1	1.7	0.204 (0.005)	1.9	2.6
8.0	8.604 (0.253)	7.6	2.9	8.061 (0.292)	0.8	3.6
16.0	15.737 (0.065)	−1.6	0.4	16.032 (0.232)	0.2	1.4
Ciprofloxacin						
0.1	0.105 (0.003)	5.1	3.2	0.105 (0.006)	4.6	6.2
0.2	0.200 (0.004)	−0.2	1.9	0.203 (0.006)	1.3	3.1
8.0	8.550 (0.228)	6.9	2.7	8.105 (0.251)	1.3	3.1
16.0	15.950 (0.200)	−0.3	1.3	16.208 (0.263)	1.3	1.6

RE: relative error, CV: coefficient of variation.

Table 4
Mean recoveries of pazufloxacin, levofloxacin, and ciprofloxacin ($n = 5$).

Nominal concentration (μg/mL)	Pazufloxacin		Levofloxacin		Ciprofloxacin	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.1	99.3	3.7	91.4	1.5	89.6	3.3
0.2	94.3	2.6	89.5	1.7	89.5	2.0
8.0	97.3	3.0	90.6	2.9	87.0	2.7
16.0	96.6	1.9	86.9	0.4	85.2	1.3

CV: coefficient of variation.

Table 5
Stability of pazufloxacin, levofloxacin, and ciprofloxacin in drug-free human serum.

Fluoroquinolone	Experimental conditions	Parameter	QC concentration (μg/mL) (n = 3)			
			0.1	0.2	8.0	16.0
Pazufloxacin	Analyzed immediately	Calculated concentration Mean (μg/mL)	0.095	0.198	7.777	16.138
		RE (%)	−5.3	−1.0	−2.8	0.9
		CV (%)	1.4	2.0	0.3	0.5
	After three freeze-thaw cycles	Calculated concentration Mean (μg/mL)	0.097	0.196	7.737	16.009
		RE (%)	−3.2	−2.2	−3.3	0.1
		CV (%)	2.0	2.4	0.9	1.2
	After storage at room temperature for 24 h	Calculated concentration Mean (μg/mL)	0.095	0.198	7.749	15.972
		RE (%)	−5.2	−1.1	−3.1	−0.2
		CV (%)	1.0	1.5	1.9	1.3
	After storage at −20 °C for three months	Calculated concentration Mean (μg/mL)	0.102	0.198	7.869	15.753
		RE (%)	2.3	−1.1	−1.6	−1.5
		CV (%)	7.0	2.8	2.6	3.0
Levofloxacin	Analyzed immediately	Calculated concentration Mean (μg/mL)	0.096	0.198	7.871	16.269
		RE (%)	−4.1	−0.8	−1.6	1.7
		CV (%)	1.0	0.8	0.4	0.8
	After three freeze-thaw cycles	Calculated concentration Mean (μg/mL)	0.096	0.196	7.808	16.107
		RE (%)	−4.3	−1.9	−2.4	0.7
		CV (%)	2.6	1.9	0.6	1.1
	After storage at room temperature for 24 h	Calculated concentration Mean (μg/mL)	0.097	0.198	7.834	16.167
		RE (%)	−3.3	−1.2	−2.1	1.0
		CV (%)	3.7	2.3	1.9	1.3
	After storage at −20 °C for three months	Calculated concentration Mean (μg/mL)	0.100	0.198	7.941	15.929
		RE (%)	0.4	−1.0	−0.7	−0.4
		CV (%)	1.2	2.4	2.5	2.9
Ciprofloxacin	Analyzed immediately	Calculated concentration Mean (μg/mL)	0.092	0.197	7.868	16.328
		RE (%)	−7.7	−1.7	−1.6	2.1
		CV (%)	0.3	1.5	0.5	1.1
	After three freeze-thaw cycles	Calculated concentration Mean (μg/mL)	0.093	0.194	7.808	16.125
		RE (%)	−6.8	−2.8	−2.4	0.8
		CV (%)	2.1	1.8	0.4	1.1
	After storage at room temperature for 24 h	Calculated concentration Mean (μg/mL)	0.093	0.196	7.832	16.211
		RE (%)	−6.6	−2.1	−2.1	1.3
		CV (%)	1.9	2.8	2.0	1.3
	After storage at −20 °C for three months	Calculated concentration Mean (μg/mL)	0.102	0.197	8.000	16.103
		RE (%)	2.2	−1.6	0.0	0.6
		CV (%)	1.5	1.4	1.1	0.7

RE: relative error, CV: coefficient of variation.

ciprofloxacin, 100.5% (0.7%). Therefore, no significant changes were present in the peak areas of analytes.

The stability of fluoroquinolones in drug-free human serum was investigated under the three conditions mentioned above. Triplicates of the QC samples at each concentration level were analyzed. As shown in Table 5, these QC samples were not observed any degradation under these three conditions.

4. Discussion

In this study, we carried out as preliminary study prior to clinical study, using human serum. This clinical study is required the simple and validated method for measuring the concentration of the three fluoroquinolones in spiked human serum. Namely, we tried to develop a new HPLC method that was able to determine simultaneously three fluorquinolone concentration.

First of all, we tried to select the concentration range of the calibration curves from previous report. Mean C_{max} of pazufloxacin in healthy adult volunteers after receiving single intravenous doses of 300 and 500 mg is reportedly 8.99 and 11.0 μg/mL, respectively [11]. Those of levofloxacin, single oral doses of 250 and 500 mg, is reportedly 2.8 and 5.7 μg/mL, respectively [32]. Mean C_{max} of ciprofloxacin, single intravenous doses of 200, 300, and 400 mg, is reportedly 2.5, 3.2, and 4.0 μg/mL, respectively [33], and single oral doses of 200, 400, and 600 mg, is reportedly 1.21, 2.45, and 3.33 μg/mL, respectively [34]. Namely, we decided that the concentration range of the calibration curves were from 0.1 to 20.0 μg/mL in this study. In fact, for all three fluoroquinolones, the within-day and between-day precisions and accuracies calculated from weighted calibration curves were acceptable (Table 3).

The three fluoroquinolones are not administered in combination with each other in the clinical setting. However, we believe that the simultaneous measuring method has some advantages in clinical settings. For example, if three patients are treated by one of the three fluoroquinolones and the serum concentration of the fluoroquinolone in each patient is to be determined, then we need to create calibration curves for three different drugs, which is a lengthy process. However, in this study, we proposed a new method by which calibration curves can be simultaneously obtained for three agents. That is, using this method, we will be able to obtain three different calibration curves quickly. This would allow quick assessment of fluoroquinolone serum concentrations in patients and enable doctors to rapidly alter dosing regimens based on

patient fluoroquinolone pharmacokinetics and MICs against bacteria. As discussed above, we believe that this method has great clinical advantages, although the three fluoroquinolones are virtually never co-administered.

We used 0.1 M HCl to prepare the stock standard solution (2 mg/mL) of ciprofloxacin in this study, since ciprofloxacin does not completely dissolve in water. The concentration of HCl used in this study was similar to that of previous reports [25,29]. The effects of 0.1 M HCl on pazufloxacin and levofloxacin were tested by comparing the differences between the peak area of standard solution diluted with 0.1 M HCl and with water. No significant change in the peak areas of both fluoroquinolones was observed. The effect of 0.1 M HCl on the determination of pazufloxacin and levofloxacin was minimal.

We tried whether the peaks of pazufloxacin, ciprofloxacin, and levofloxacin were able to separate or not. The mobile phase used in this study did not contain ion-pair reagents. Several previous articles have been reported about the simultaneous quantification methods of two or more fluoroquinolones in human plasma using HPLC [28–30]. These mobile phases were contained ion-pair reagents such as tetrabutylammonium salt and sodium dodecyl sulfate, and which might lead to problems in column maintenance [35]. The present method was not induced these problems, because of the absence of ion-pair regents in the mobile phase. It is considered that this advantage is favorable for clinical routine applications. In this study, the fluoroquinolones were separated using a C_8 column (Fig. 2a). The method for simultaneously measuring four fluoroquinolones including ciprofloxacin in human serum using a C_8 column has been reported previously [27]. Before using a C_8 column, we carried out the separation of fluoroquinolones using an Inertsil® ODS-SP column (250 mm × 4.6 mm i.d., particle size 5 μm; GL Sciences). The mobile phase was 1% triethylamine (pH 3.0)/acetonitrile (86/14, v/v). For the measurement of fluoroquinolones, many researchers had used the C_{18} column [22–26,28–30]. It was confirmed that for the peak of pazufloxacin and levofloxacin, separation with the C_8 column was better than that with the C_{18} column. In addition, separation with the C_8 column for ciprofloxacin was equivalent to that with the C_{18} column. From these results, we decided to use C_8 column for separation of fluoroquinolones. The chemical structure of pazufloxacin and levofloxacin differs at the C-10 position of the 7-oxopyrido-[1,2,3-de][1,4]-benzoxazine-6-carboxylic acid. Pazufloxacin and levofloxacin include at C-10 position a 1-aminocyclopropyl group and a 4-methylpiperazinyl group, respectively. It may be thought that when the C_8 column is used, pazufloxacin and levofloxacin easily interact with the silanol groups of the stationary phase since the C_8 column possesses less steric hindrance than the C_{18} column in the surface of the silica gel stationary phase. We believe that the difference in these chemical structures at the C-10 position and steric hindrance in the surface of the silica gel stationary phase may influence the separation of pazufloxacin and levofloxacin.

We carried out protein precipitation by adding 6.0% (w/v) perchloric acid and methanol. Several investigators have reported the HPLC method for determination of ciprofloxacin and trovafloxacin in human serum or plasma, which involves protein precipitation with a mixture of perchloric acid and acetonitrile [26,36,37]. To develop the protein precipitation procedure, we tested the change of sample preparation by adding organic solvents (methanol, ethanol, and acetonitrile) or 6.0% (w/v) perchloric acid to equal amounts of serum. When using methanol, it was not able to obtain a sufficiently clear supernatant. Furthermore, when using acetonitrile or ethanol, the peaks of fluoroquinolones became either very broad or very small. A protein precipitation using 6.0% (w/v) perchloric acid yielded a clear supernatant, however, the recovery of ciprofloxacin was relatively low (about 75%). Furthermore, to improve this low recovery, we tried to add an organic solvent (acetonitrile or methanol). Eventually, when using 6.0% (w/v) perchloric acid and methanol, we could get the highest recovery of fluoroquinolones. When this protein precipitation method was used, the recoveries of the three fluoroquinolones and its CV% were satisfactory (Table 4). It is considered that some part of the ciprofloxacin molecules which co-precipitated with serum protein is extracted from the pellet into the supernatant by adding methanol. This may improve the recovery of ciprofloxacin. The sample preparation method in this study is faster and simpler than methods involving evaporation steps [23,27,29], ultrafiltration requiring long centrifugation times [22,28], and liquid–liquid extraction [38]. It was considered that our sample preparation method is suitable for routine clinical applications.

In this study, an HPLC method with fluorescence detection for simultaneous quantification of pazufloxacin, ciprofloxacin, and levofloxacin in spiked human serum was developed and validated. Pazufloxacin, ciprofloxacin, and levofloxacin were considered to be stable in drug-free human serum after three freeze-thaw cycles, after storage at room temperature for 24 h, and after storage at −20 °C for three months. This HPLC method involved simple sample preparations enabled quantification of these three fluoroquinolones in spiked human serum without changing the analytical conditions. In future, this method will help in clinical study and routine analysis.

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