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Improvement and validation of an HPLC method for examining the effects of the *MDR1* gene polymorphism on sparfloxacin pharmacokinetics

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Abstract

A rapid, simple, accurate, and precise reverse-phase high-performance liquid chromatography (HPLC) method for measuring sparfloxacin in human serum was improved, validated, and applied to determine the influence of polymorphisms in *MDR1* (exons 12, 21, and 26) gene on sparfloxacin pharmacokinetics. Sparfloxacin and an internal standard, ciprofloxacin, were extracted from human serum by protein precipitation with dilution and analyzed on a Luna C_{18} 5-μm column in a mobile phase of acetonitrile–0.035 M perchloric acid (28:72, v/v, adjusted to pH 2.0 with 0.015 M triethylamine) and UV detection at 300 nm. This analysis was performed at three different laboratories using the same quality control (QC) samples. The chromatograms showed good resolution, sensitivity, and no interference by human serum. The method showed linear responses over a concentration range of 0.05–2 μg/ml, with correlation coefficients of greater than 0.999 at the three laboratories. Intra- and inter-day assay precision and accuracy fulfilled international requirements. The mean absolute recovery for human serum was 98.8 ± 5.7%. Sparfloxacin in human serum was stable during storage and the assay procedure. The lower limit of quantification using 0.2 ml of serum was 0.05 μg/ml, which was sensitive enough for pharmacokinetic studies. This method was used to study the pharmacokinetics of sparfloxacin in human volunteers, following a single oral administration of sparfloxacin (100 mg) two tablets at three different laboratories. *MDR1* polymorphisms did not significantly ($P < 0.01$) affect the pharmacokinetic parameters (AUC and C_{max}) of sparfloxacin.

Keywords: Sparfloxacin; *MDR1*; Pharmacokinetics

1. Introduction

Sparfloxacin, 5-amino-1-cyclopropyl-6,8-difluoro-1,4-dihydro-7-(cis-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid (Fig. 1), is a synthetic, broad-spectrum fluoroquinolone antimicrobial agent for oral administration. Sparfloxacin, a potent inhibitor of bacterial DNA gyrase, is bactericidal at or near the minimum inhibitory concentration [1]. Sparfloxacin is effective against Gram-positive and Gram-negative bacteria and is more active against some micro-organisms, including staphylococci and mycobacteria, in vitro than other quinolones [2]. It is used in the treatment of community-acquired pneumonia and acute bacterial exacerbations of chronic bronchitis in adult patients [3,4]. Sparfloxacin has an absolute oral bioavailability of 92%. Maximum concentrations in plasma are observed 3–5 h after oral administration [4]. Sparfloxacin shows a long elimination half-life of 16–20 h, moderate protein binding (45%), excellent tissue distribution, and effective penetration into extracellular fluids [5,6]. The only metabolite of sparfloxacin isolated in man is sparfloxacin glucuronide, which is excreted in urine by both glomerular filtration and tubular secretion [1]. Unlike other fluoroquinolones, metabolism of sparfloxacin is not cytochrome P-450-dependent. Instead, elimination of sparfloxacin is through a combination of renal, biliary, and transintestinal secretion [4].

Sparfloxacin is a *P*-glycoprotein (*P*-gp) substrate both in vitro and in vivo in mice [7,8]. *P*-gp is one of the most important transporters for the disposition of drugs and xenobiotics. *P*-gp acts as an apical (luminal) exporter in organs and tissues with excretory or barrier functions (e.g. intestine, liver, kidney, the blood–brain barrier, and the blood–testes barrier) and influences

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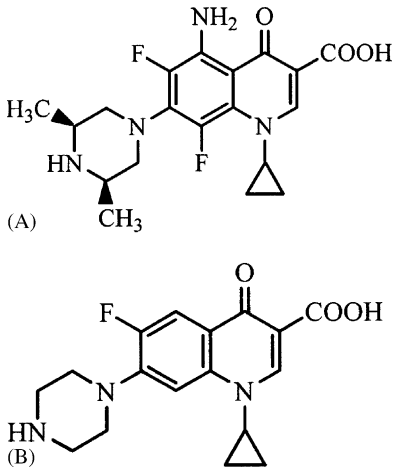

Fig. 1. Chemical structures of sparfloxacin and ciprofloxacin (I.S.).

the absorption, distribution, and excretion processes. Expression levels and the function of *P*-gp directly affect plasma levels and intracellular concentrations of drugs and thereby influence therapeutic responses [9,10]. Several single-nucleotide polymorphisms (SNPs) exist in the human *MDR1* gene, which codes for *P*-gp. Allelic variations in exons 12, 21, and 26 of the *MDR1* gene were associated with altered *P*-gp function [11].

Previous analysis of sparfloxacin was based on the microbiological agar well method [12], high-performance thin-layer chromatography (HPTLC) [13], and high-performance liquid chromatography (HPLC) with UV [14] or fluorescence detection [15]. These methods use a relatively large plasma volume [13,15] and injection volume [14,15] or include time-consuming solvent evaporation [13,15] and relatively longer analytical time (more than 15 min) [14]. Moreover, there are no reports showing the robustness of an analytical procedure of sparfloxacin and the effects of *MDR1* polymorphisms on sparfloxacin pharmacokinetics.

Therefore, we sought to improve and validate a simple and rapid HPLC method with UV detection for assessing the pharmacokinetics of sparfloxacin in a routine setting with respect to the genetic polymorphism in *MDR1* gene. To develop this method, we changed the flow rates and organic solvent composition of the mobile phase and tested the same quality control (QC) samples at three different laboratories. We also examined the effects of the major *MDR1* polymorphisms on sparfloxacin pharmacokinetics.

2. Experimental methods

2.1. HPLC analysis

2.1.1. Chemicals and reagents

Sparfloxacin (>99.5% purity) and ciprofloxacin hydrochloride (internal standard, 99.8% purity, Fig. 1) were kindly supplied by Sama Pharm. Co. (Seoul, Republic of Korea). Methanol and acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, NJ, USA), and other chemicals were of HPLC grade or the highest quality available. HPLC grade water was obtained from a Milli-Q water purification system (Millipore Co., Milford, MA, USA) and used throughout the study. The mobile phase components such as acetonitrile, perchloric acid, and deionized water were filtered through a 0.45-μm pore-size membrane filter prior to mixing.

2.1.2. Instruments and chromatographic condition

The HPLC system was obtained from Shimadzu Corporation (Kyoto, Japan). It consisted of two pumps (model LC-10AD), a degasser (model DGU-12A), and a UV detector (model SPD-10A*vp*) set at 300 nm. The detection wavelength, 300 nm, was determined by scanning the maximum absorbance wavelength of sparfloxacin in mobile phase with UV spectrophotometer (Uvikon 930, Kontron Instruments, Zürich, Switzerland). Test samples were injected using a model 7725i injector (Rheodyne, Cotati, CA, USA). The separation was performed on a Luna $C_{18}(2)$ column (5-μm particle size, 250 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) at ambient temperature. The mobile phase was a mixture (28:72, v/v) of acetonitrile and 0.035 M perchloric acid adjusted to pH 2.0 with 0.015 M triethylamine and was prepared daily. The flow rate was 0.8 ml/min for a total running time of 10 min. Detector output was quantified on a model Class LC-10 integrator (Shimadzu, Kyoto, Japan).

2.1.3. Stock solutions and standards

Stock solutions of sparfloxacin (1 mg/ml) and ciprofloxacin hydrochloride (1 mg/ml as base) were prepared in methanol-distilled water (1:10) and stored in light-protected glass bottle at 4 °C. Serum calibration standards of sparfloxacin were freshly prepared daily at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml in drug-free pooled serum obtained from 12 different volunteers. In the same manner, quality control serum samples at low (0.2 μg/ml), medium (1 μg/ml), and high (10 μg/ml) were freshly prepared daily to evaluate accuracy and precision. All solutions and samples of sparfloxacin and ciprofloxacin were protected against light during manipulations.

2.1.4. Extraction procedures

To 200 μl of blank serum, calibration standards and QC samples in 1.7 ml MaxyClear microtubes (amber polypropylene, MCT-175-X, Axygen Scientific, Inc., Union City, CA, USA), 100 μl of I.S. (ciprofloxacin, 5 μg/ml), and 200 μl of a solution (0.5 g $ZnSO_4$ dissolved in 100 ml methanol, plus 1 ml ethylene glycol for the purpose of increasing precipitation efficiency) were added. The samples were extracted by vortex-mixing for 30 s at high speed and centrifuged at 10,000 × *g* for 10 min. The aqueous layer (upper phase) was transferred into a 1.7 ml MaxyClear microtube and diluted with an equal amount of distilled water, and 10 μl of the solution was then injected onto the HPLC system.

2.2. HPLC method validation

2.2.1. Specificity

The interference of endogenous compounds in serum was assessed by analyzing standard sparfloxacin, drug-free serum, serum spiked with sparfloxacin, and serum samples obtained

from subjects given sparfloxacin. All peaks showing the retention time of sparfloxacin and ciprofloxacin were analyzed with a diode array detector (2017 Diode Array, Shiseido, Tokyo, Japan).

2.2.2. Sensitivity

The lower limit of quantitation (LLOQ) was defined as the lowest concentration yielding a precision with less than 20% CV and accuracy between 80 and 120% of the theoretical value. The LLOQ was 0.05 μg/ml for sparfloxacin in nine replicates.

2.2.3. Linearity

The linearity of the calibration curve for sparfloxacin was assessed in the range of 0.05–20 μg/ml in serum. Samples were quantified using the ratio of peak area of analyte to that of I.S. Peak area ratios were plotted against serum concentrations, and the straight-line regression equation was weighted (weighting factor: 1/concentration) and presented with its correlation coefficient (r).

2.2.4. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, QC samples were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV) for the analysis of QC samples in five replicates, and inter-day precision was determined by the analysis of QC samples on five consecutive days. Accuracy was determined by comparing the calculated and known concentrations. Accuracy and precision were evaluated at three different laboratories using the same QC samples.

The evaluation of precision was based on the criteria that relative standard deviation for each concentration level should not be more than ±15% except for the LLOQ, for which it should not exceed ±20%. Similarly, for accuracy, the mean value should not deviate by ±15% of the nominal concentration except for the LLOQ, where the limit was ±20%.

2.2.5. Recovery

The absolute recoveries from human serum were assessed by comparison of the peak area from extracted QC samples to the peak area of the corresponding standards, followed by application of a correction factor (correction factor = 5). Relative recoveries from human serum were assessed by comparison of the peak area from extracted QC samples to those from extracted samples in water instead of serum. The mean recoveries were determined at low, medium, and high concentrations in five replicates.

2.2.6. Stability

To test the short- and long-term stability of sparfloxacin, two QC samples, low (0.2 μg/ml) and high (10 μg/ml), were stored in light-protected storage boxes under different conditions: at room temperature for 0, 4, and 24 h and at −80 °C for 1 month. To test the stability of stock solutions stored in the light-protected glass bottles at 4 °C, they were diluted with mobile phase and injected onto the HPLC system at predetermined time intervals of up to 1 month. Three freeze–thaw cycles (−80 °C/room temperature) in dark condition were applied to the above QC samples on three consecutive days.

The compounds were considered stable if assay variation ($n = 5$) was less than 10% of initial time response.

Table 1
Factorial design for 3-level-2-factor investigation in the robustness test

Factors	Levels		
	Low (−1)	High (1)	Normal (0)
Flow rate of the mobile phase (ml/min)	0.6	1.0	0.8
Organic solvent composition (%) in the mobile phase (ACN:0.035 *M* perchloric acid with 0.015 *M* triethylamine)	26:74	30:70	28:72

2.2.7. Robustness

Robustness can be defined as the ability to reproduce the analytical method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained results [16,17]. Assay robustness for small changes of acetonitrile content (26–30%) and flow rate (0.6–1.0 ml/min) was confirmed by a 3^2 factorial design and a fixed sparfloxacin concentration (1 μg/ml), analyzed with respect to its peak area and retention time (Table 1).

Also, analytical robustness was studied during the assay qualification by replicating qualification tests in three different laboratories: laboratory 1, Institute of Bioequivalence and Bridging Study, College of Pharmacy, Chonnam National University; laboratory 2, Department of Pharmaceutics, College of Pharmacy, Chosun University; laboratory 3, Clinical Trial Center, Chonnam National University Hospital. Each laboratory performed identical sequences of test analyses, including spike/recovery and standard sparfloxacin linearity determinations. Chromatographic sequences in three laboratories included identical standards. Each laboratory utilized an identically configured Shimadzu HPLC system including the same batch Luna $C_{18}(2)$ column (B/N 5291-57), and testing was performed by three analysts.

2.3. Influence of MDR1 polymorphisms on sparfloxacin pharmacokinetics

2.3.1. Subjects

A total of 426 healthy Korean volunteers were genotyped for the *MDR1* exon 12 C1236T, exon 21 G2677T/A (Ala893Ser/Thr), and exon 26 C3435T polymorphisms. Of these volunteers, 24 subjects (7 females and 17 males), 8 subjects per institute, also participated in the pharmacokinetic study of sparfloxacin. Their age was 24.17 ± 3.51 years (mean ± S.D., range: 20–37 years), body weight was 62.88 ± 6.56 kg, and mean height was 170.88 ± 7.01 cm. All volunteers had given informed written consent to undergo genotyping and pharmacokinetic studies. The study protocol was approved by each Institutional Review Board (IRB). This study was performed

according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of Good Clinical Practice. The subjects were determined to be in good health before the study on the basis of physical examination, medical history, and laboratory tests.

The subjects were classified into three groups on the basis of *MDR1* genotype for the C3435T polymorphisms. The first group included 8 wild-type carriers of the GG genotype, the second group contained 10 heterozygous carriers of the GT, and the third group included 6 homozygous TT carriers.

2.3.2. MDR1 genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the Wizard Genomic DNA purification kit (Promega Co., Madison, WI, USA). Candidate SNPs (C1236T, G2677T/A, and C3435T) in the *MDR1* gene were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [9,11]. PCR was carried out in a 20 μl reaction mixture containing PCR PreMix, 200–300 ng of genomic DNA and 10 pmol of each primer. The PCR conditions consisted of an initial denaturation step for 2 min at 94 °C, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 59.8 °C for 30 s, and extension at 72 °C for 30 s. The terminal extension was performed at 72 °C for 5 min. After amplification of the specific fragments, SNPs were detected by digestion with specific restriction enzymes capable of discriminating between wild type and mutant alleles. The digestion PCR products were then separated on 2–2.5% agarose gels of varying concentrations depending on size, and visualized on an UV transilluminator after ethidium bromide staining. To acquire exact genotype data, we conducted experiments three times.

2.3.3. Study design

After overnight fasting, each subject received a single oral dose of sparfloxacin (two 100 mg tablets) with 240 ml of water. Food was taken after 4 h, and consumption of alcohol or xanthine-containing foods and beverages was restricted during the study.

Sampling was identical for all the subjects. Blood samples were collected in Vacutainer® (5 ml, Becton Dickinson and Company, Franklin Lakes, NJ, USA) tubes before dosing and at 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, and 72 h after drug administration. Following centrifugation (3000 × *g*, 20 min, 4 °C), serum samples were transferred to polyethylene tubes and immediately stored at −80 °C until analysis. Care was taken against too much exposure to light. This study was sequentially performed at three institutes with an identical protocol. The serum samples were analyzed for sparfloxacin content by the proposed HPLC method.

2.3.4. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by non-compartmental analysis of serum concentration-time curve data using WinNonlin software (Pharsight Corporation, CA, USA) [18]. The peak concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined from individual serum concentration-time profiles for sparfloxacin. The area under the serum concentration-time curve (AUC_{0-t}) was calculated by the linear trapezoidal rule from 0 to 72 h. The area under the serum concentration-time curve from zero to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/\lambda_Z$, where C_t is the last measurable concentration. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_Z$, where λ_Z is terminal rate constant.

2.3.5. Statistical analysis

Allele and genotype frequencies for the various SNPs were assessed for deviation from Hardy–Weinberg equilibrium using Pearson's chi-square test. Samples in which one or two loci could not be genotyped were excluded from haplotype frequency calculations. Estimation of haplotype frequencies for *MDR1* was performed by maximum likelihood estimation, based on the expectation-maximization (EM) algorithm using the population genetics data analysis program Arlequin [19].

Data from three or more different genotype groups were compared with the Kruskal–Wallis test or ANOVA with the Tukey–Kramer multiple-comparison test. A P-value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS statistical software.

3. Results and discussion

3.1. Chromatography

Serum chromatograms of oral sparfloxacin (200 mg) are shown in Fig. 2C. The retention times for sparfloxacin and I.S. were 8.0 and 4.5 min, respectively. The Luna $C_{18}(2)$ column with the mobile phase of acetonitrile and 0.035 M perchloric acid (pH 2.0) resulted in the short chromatographic run time (10 min), which was more shorter than that (15 min) reported by Kamberi et al. [14]. The mobile phase used for the assay was of very simple composition, was inexpensive, and achieved optimal resolution of sparfloxacin and the I.S. with no interference from other components in serum. The chromatographic runs indicated that the acidic mobile phase with triethylamine as a modifier improved chromatographic characteristics (peak symmetry proved to be better). By contrast, the sample preparation was even simpler and there was an appreciable reduction in the procedural time. Another important characteristic of our method was the precipitation solution that we chose. The fact that we had no interference from endogenous serum substances is probably due to the use of the precipitation mixture of methanol, $ZnSO_4$, and ethylene glycol. As demonstrated, this mixture permits a good recovery of the compounds of interest. Moreover, we could analyze more than 200 samples on the same column without any deterioration of its performance by injection of 10 μl solution.

3.2. HPLC method validation

3.2.1. Specificity

Fig. 2 shows representative chromatograms of the human serum with extraction using the mixture of methanol, $ZnSO_4$, and ethylene glycol. No peaks interfered with sparfloxacin and I.S. at their retention times in blank human serum (Fig. 2). We

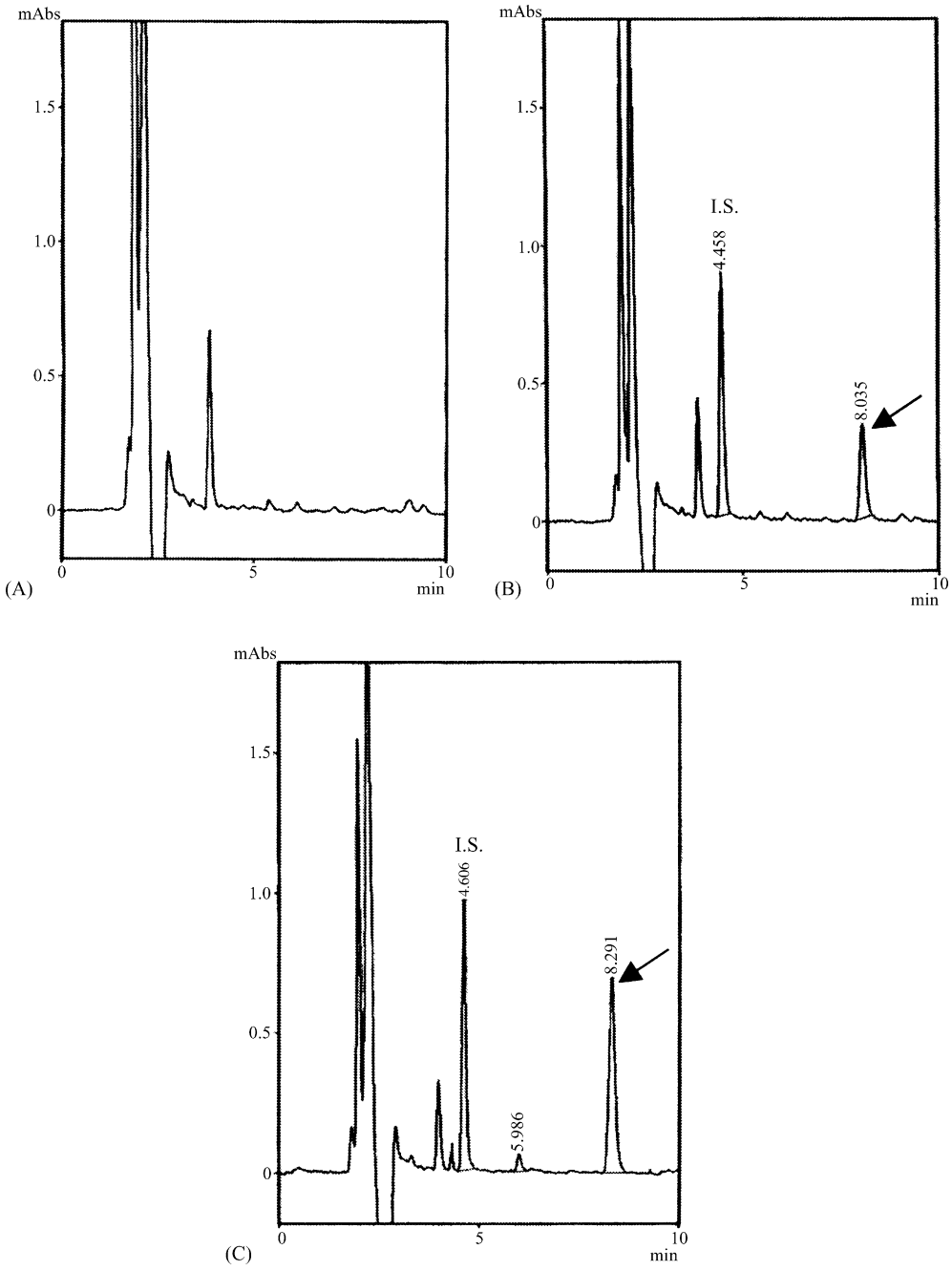

Fig. 2. Chromatograms of: (A) blank human serum; (B) blank human serum spiked with sparfloxacin (0.5 μg/ml) and internal standard (I.S., ciprofloxacin 2.5 μg/ml); and (C) serum sample (0.95 μg/ml) at 3 h after oral administration of two Spara tablets (sparfloxacin 100 mg). (↙) sparfloxacin peak.

could confirm identical peak spectra with the diode array detector.

3.2.2. Sensitivity

The lower limit of quantitation (LLOQ) was defined as those quantities that were 10-fold of the background noise, with precision errors of less than 20% (CV) and inaccuracy between ±20% (bias). The LLOQ for 200 μl of human serum was found to be 0.05 μg/ml for sparfloxacin-injected on-column with a 10 μl loop. The mean percent accuracy value for serum samples was 95.24% and precision coefficient of variation (CV) was below 9.69% at the LLOQ (Table 2). The LLOQ value for serum was the same as that reported by Mody et al. [13] and Borner et al. [15] and 2-fold higher than that reported by Kamberi et al. [14]. However, those methods used a relatively large plasma volume [13,15] and injection volume [14,15]. Basically, if we use the same injection volume, we can achieve the LLOQ value up to 2.5-fold lower than that reported by Kamberi et al. [14].

3.2.3. Linearity

The calibration curves for sparfloxacin were linear over the concentration range of 0.05–20 μl/ml in human serum. The mean (±S.D.) regression equations from nine replicate calibration curves on different days for human serum at three dif-

Table 2
Precision and accuracy for the determination of sparfloxacin in human serum at each institute

Concentration (μg/ml)	Precision CV (%)						Accuracy (%)					
	Intra-day ($n=5$)			Inter-day ($n=5$)			Intra-day ($n=5$)			Inter-day ($n=5$)		
	1st[a]	2nd[a]	3rd[a]	1st[a]	2nd[a]	3rd[a]	1st[a]	2nd[a]	3rd[a]	1st[a]	2nd[a]	3rd[a]
0.05 (LLOQ)	9.10	8.45	4.47	9.69	6.89	3.08	87.85	110.94	84.51	84.02	113.17	83.36
0.2 (low)	5.87	4.94	5.60	5.62	3.86	4.16	95.78	105.98	85.96	93.73	106.31	88.74
1 (medium)	4.80	2.29	1.64	5.75	2.89	2.73	99.88	100.00	99.17	99.82	99.92	99.96
10 (high)	8.12	1.61	2.79	4.51	2.36	1.94	100.45	98.93	103.28	97.28	100.69	101.76

[a] Institutes.

ferent laboratories, $y=(1.1747\pm0.0787)x+(0.0057\pm0.0005)$ at 1st institute; $y=(1.1395\pm0.0677)x-(0.0036\pm0.0003)$ at 2nd institute; $y=(1.1910\pm0.0598)x-(0.0049\pm0.0004)$ at 3rd institute (where, y = peak-area ratio, x = concentration), showed significant linearities ($r=0.9997\pm0.0005$, $r=0.9996\pm0.0004$, $r=0.9993\pm0.0004$, respectively) with statistically insignificant ($P>0.001$) nonlinear elements in the residual sum of squares, as determined by analysis of variance.

3.2.4. Precision and accuracy

Table 2 shows a summary of intra- and inter-day precision and accuracy. In the range of 0.05–20 μl/ml, intra- and inter-day accuracy ranged from 84.51 to 110.94 and 83.36 to 113.17%, respectively. The intra- and inter-day assay precision (CV) ranged from 1.61 to 9.10 and 1.94 to 9.69%, respectively. These results indicate that the present method has a satisfactory accuracy, precision, and reproducibility.

3.2.5. Recovery

The extraction recoveries of sparfloxacin were determined at low (0.2 μg/ml), medium (1 μg/ml), and high (10 μg/ml) concentrations in five replicates. The mean absolute recoveries of sparfloxacin and ciprofloxacin from human serum were 99.2 ± 3.7 and 94.2 ± 5.2%, respectively. The mean relative recovery of sparfloxacin from human serum was 103.9 ± 2.6%. This high, reproducible recovery of sparfloxacin was able to increase its assay sensitivity; the simple extraction procedure involving protein precipitation with the mixture of methanol, $ZnSO_4$, and ethylene glycol has been successfully applied to the extraction of sparfloxacin from human serum.

3.2.6. Stability

Two QC samples (low and high) of sparfloxacin in dark condition were stable in serum at room temperature up to 24 h, with variation of less than 8.2%. They were also stable for 1 month at −80 °C in serum (CV was less than 5.21%), and after three freeze–thaw cycles, changes in peak area were within acceptable limits (≤8.56%). Finally, the stock solutions of sparfloxacin and the I.S. stored in the light-protected glass bottles at 4 °C were stable for at least 1 month with variation of less than 10.0%.

3.2.7. Robustness

Assay robustness was evaluated by changing the flow rate (0.6–1.0 ml/min) and acetonitrile:0.035 M perchloric acid with 0.015 M triethylamine ratio in the mobile phase (26:74–30:70). To determine the effects on peak area and retention time, a fixed sparfloxacin concentration (1 μg/ml) was injected three times after each change. Preliminary inspection of chromatograms obtained under these various conditions suggested that the method is fairly robust. Minor changes in flow rate and solvent composition did not have any significant ($P<0.05$) effect on peak area and retention time (Table 3).

Results from three laboratories using the same HPLC instruments (Shimadzu LC 10 AD system) showed no significant

Table 3
Analysis of variance for the factorial design of robustness test based on peak area and retention time

Factors	Mean square	F	P
Peak area			
Flow rate (flow)	3.91×10^4	1.71	0.290
Mobile phase composition (MP)	3.87×10^4	1.70	0.293
Flow × MP	2.28×10^4	0.759	0.566
Retention time			
Flow rate (flow)	0.014	0.723	0.539
Mobile phase composition (MP)	0.044	2.215	0.225
Flow × MP	0.020	1.226	0.335

Table 4
Allele and genotype frequencies in the *MDR1* gene

Exon	Genotype frequency (%)		Allele frequency (%)	
12 C1236T	CC	20.19	C	43.43
	CT	46.48	T	56.57
	TT	33.33		
21 G2677T/A	GG	30.75	G	55.63
	GT	42.25	T	34.51
	TT	9.86	A	9.86
	GA	7.51		
	TA	7.04		
	AA	2.58		
26 C3435T	CC	38.73	C	63.85
	CT	50.24	T	36.51
	TT	11.03		

Table 5
Pharmacokinetic parameter values for each institute obtained after oral administration of sparfloxacin (mean ± S.D.)

Parameters	1st Institute ($n=8$)	2nd Institute ($n=8$)	3rd Institute ($n=8$)	Total ($n=24$)
AUC_{0-4} (μg h/ml)	2.88 ± 0.90	2.43 ± 0.98	2.72 ± 1.12	2.68 ± 0.98
$AUC_{0-\infty}$ (μg h/ml)	27.57 ± 6.46	27.17 ± 7.31	26.25 ± 1.78	27.00 ± 5.50
C_{max} (μg/ml)	1.00 ± 0.26	1.00 ± 0.30	0.99 ± 0.20	0.99 ± 0.25
T_{max} (h)	4.38 ± 2.26	4.50 ± 2.45	3.75 ± 1.28	4.21 ± 2.00
$t_{1/2}$ (h)	17.77 ± 2.78	19.42 ± 2.65	17.74 ± 2.95	18.31 ± 2.79

($P<0.05$) differences in peak areas of sparfloxacin and I.S. The retention times of sparfloxacin and I.S. ranged from 7.8 to 8.3 min and 4.2 to 4.8 min, respectively. A small change in sparfloxacin retention time was observed in different laboratories, but peak separation was still sufficient for quantitation. The intra- and inter-day CV for precision and accuracy from QC samples were less than 15% on each laboratory. It can be concluded that this method is reproducible in different laboratories by different operators under the condition of the same batch of column and LC system.

3.3. Pharmacokinetics of sparfloxacin in human and correlations of MDR1 polymorphisms

3.3.1. Genotyping and haplotype analysis

For all of the 426 individuals analyzed, the allele frequencies of *MDR1* 1236T, 2677T, 2677A, and 3435T were 56.57, 34.51, 9.86, and 36.15%, respectively (Table 4).

All of the possible twelve haplotypes were observed, and three *MDR1* haplotypes (CGC, TTT, and TGC) were observed with higher frequencies in Koreans. Three major haplotypes,

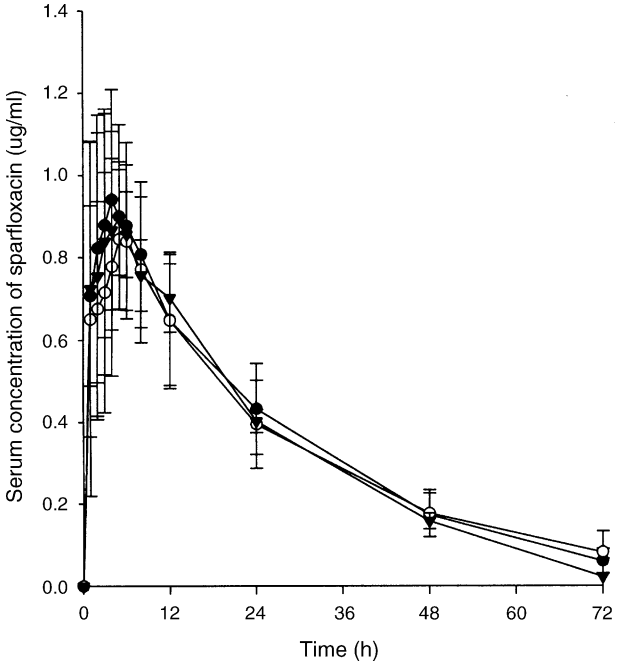

Fig. 3. Mean (±S.D.) serum concentration-time curves of sparfloxacin for each institute following oral administration of two Spara tablets (sparfloxacin 100 mg). Keys: (●) 1st institute ($n=8$); (○) 2nd institute ($n=8$); and (▲) 3rd institute ($n=8$).

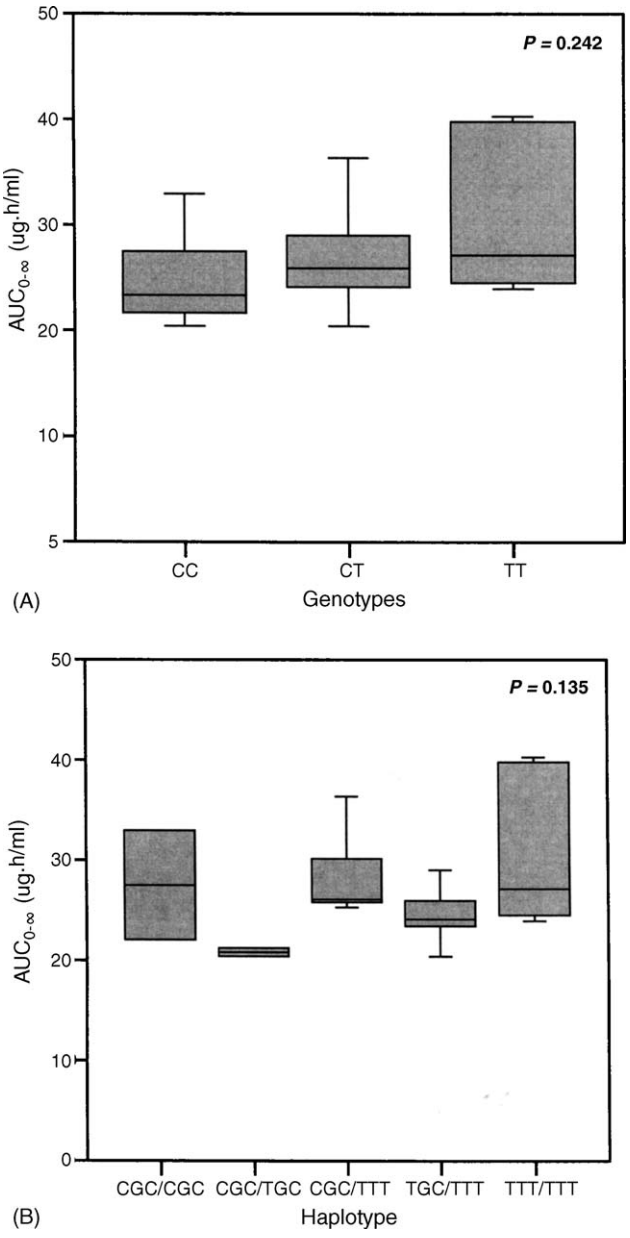

Fig. 4. (A) Values for area under concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$) after oral administration of sparfloxacin 200 mg, with regard to dependence of *MDR1* genotype C3435T in exon 26. (B) Values for $AUC_{0-\infty}$ after oral administration of sparfloxacin 200 mg, with regard to dependence of *MDR1* polymorphisms with combinations of C1236T in exon 12, G2677T in exon 21, and C3435T in exon 26. Plots depict the median and 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars.

CGC, TGC, and TTT, constituted 29.96, 20.06, and 29.49% of all haplotypes, respectively.

3.3.2. Pharmacokinetic results

We used this method in a pharmacokinetic study of a single oral dose of sparfloxacin (200 mg) in 24 healthy Korean subjects. The pharmacokinetic study was performed with identical protocols at the three institutes. All subjects completed the study and tolerated the protocol well.

Sparfloxacin pharmacokinetic parameters for each institute are provided in Table 5. Fig. 3 shows the mean (±S.D.) serum concentration-time curves of sparfloxacin in 24 subjects. Mean values (±S.D.) of C_{max}, T_{max}, $AUC_{0-\infty}$, and $t_{1/2}$ were 0.99 ± 0.25 μg/ml, 4.21 ± 2.00 h, 27.00 ± 5.50 μg h/ml, and 18.31 ± 2.79 h, respectively, and were similar to reported values, 0.70 ± 0.16 μg/ml, 4–5 h, 18.75 ± 2.93 μg h/ml, and 20.79 ± 3.62 h, respectively [20]. Gender differences in the pharmacokinetics of sparfloxacin were not observed.

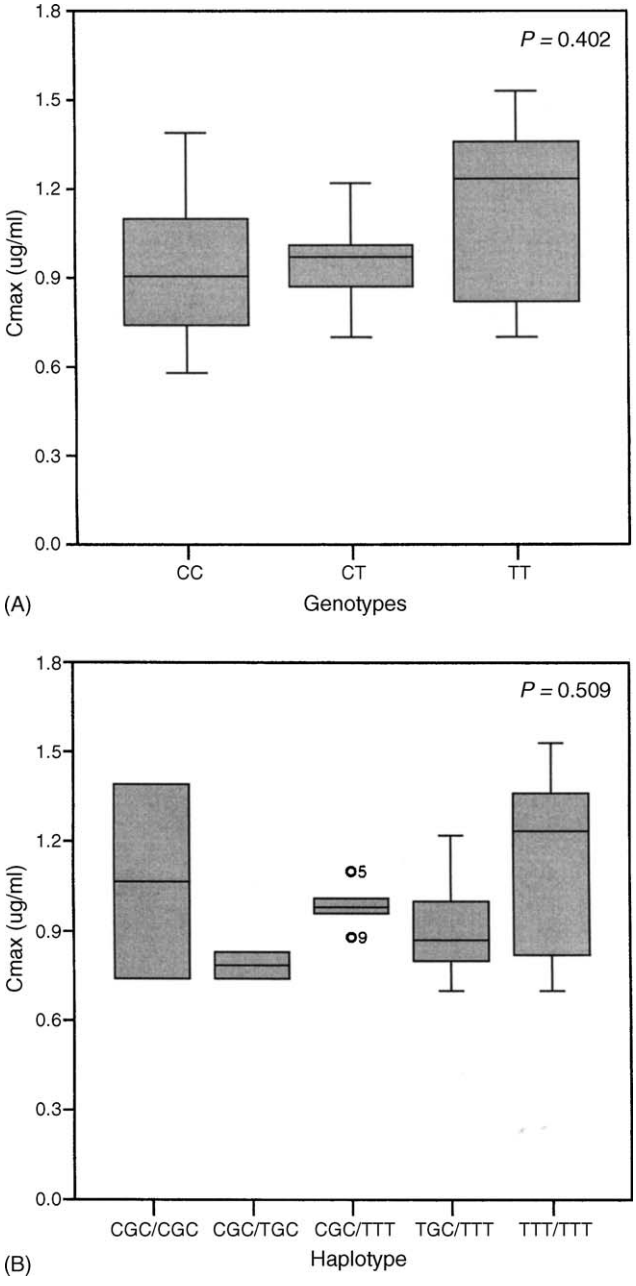

Fig. 5. (A) Values for C_{max} after oral administration of sparfloxacin 200 mg, with regard to dependence on the *MDR1* genotype C3435T in exon 26. (B) Values for C_{max} after oral administration of sparfloxacin 200 mg, with regard to dependence of combinations of *MDR1* polymorphisms. Plots depict the median and 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars.

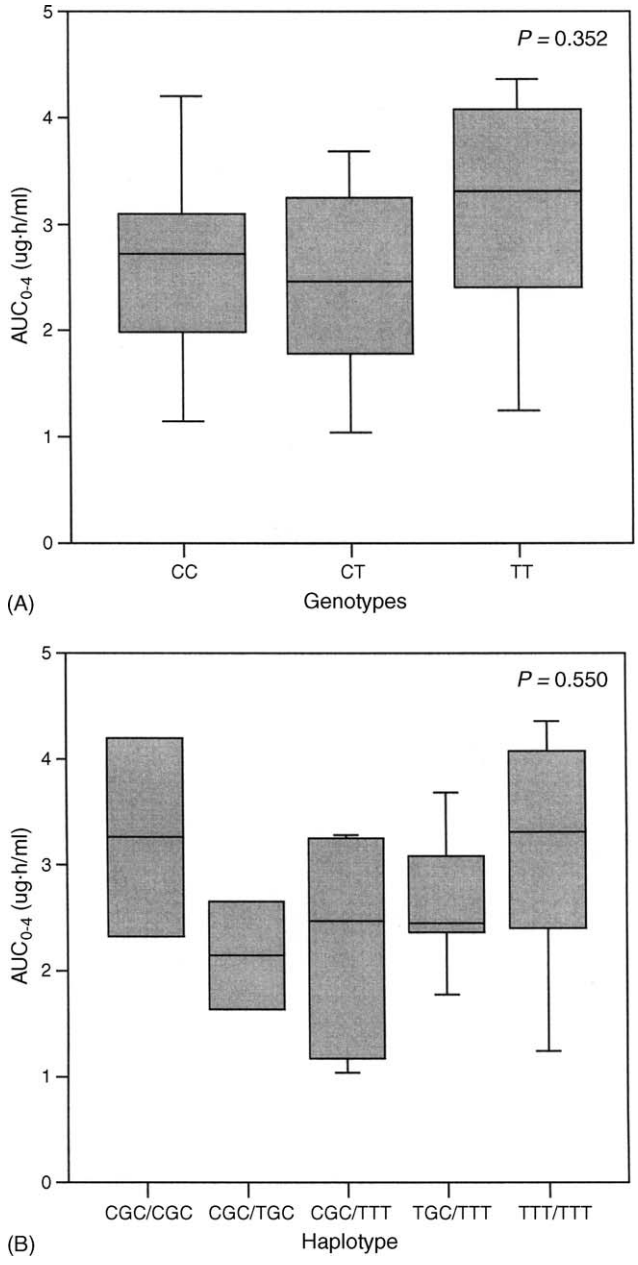

Fig. 6. (A) Values for area under concentration-time curve from time 0 to 4 h (AUC_{0-4}) after oral administration of sparfloxacin 200 mg, with regard to dependence of *MDR1* genotype C3435T in exon 26. (B) Values for AUC_{0-4} after oral administration of sparfloxacin 200 mg, with regard to dependence of *MDR1* polymorphisms with combinations of C1236T in exon 12, G2677T in exon 21, and C3435T in exon 26. Plots depict the median and 10th, 25th, 75th, and 90th percentiles as vertical boxes with error bars.

3.3.3. Effects of MDR1 genotype on disposition of sparfloxacin

We examined the relationship between each *MDR1* SNP and sparfloxacin pharmacokinetic parameters ($AUC_{0\text{-}4}$, $AUC_{0-\infty}$ and C_{max}). The 24 subjects revealed considerable pharmacokinetic variability. AUC from time 0 to 4 h (AUC_{0-4}), AUC from time 0 to infinity ($AUC_{0-\infty}$), and C_{max} ranged between 1.04 and 4.36 μg h/ml (4.19-fold), 20.41 and 40.27 μg h/ml (1.97-fold), and 0.58 and 1.53 μg/ml (2.64-fold), respectively.

Although there were roughly two- to four-fold interindividual variations within each genotype, we did not identify any significant ($P < 0.05$) differences between genotypes and pharmacokinetic parameters. However, there was a trend toward higher oral sparfloxacin $AUC_{0-\infty}$ and C_{max} in C3435T subjects. Figs. 4A and 5A show that subjects with 3435TT genotype had higher $AUC_{0-\infty}$ and C_{max} of sparfloxacin compared with subjects who were homozygous 3435CC. After administration of a single oral dose of 200 mg sparfloxacin, the pharmacokinetics of the absorptive phase characterized by AUC_{0-4} was also not significantly ($P < 0.05$) different between the genotype groups (Fig. 6A).

We also examined the correlation between the *MDR1* haplotypes (exons 12, 21, and 26) and sparfloxacin pharmacokinetics (AUC_{0-4}, $AUC_{0-\infty}$, and C_{max}), but we did not identify any correlations between the *MDR1* haplotypes and $AUC_{0-\infty}$ or any other pharmacokinetic characteristics (Figs. 4B, 5B and 6B).

4. Conclusion

A rapid, simple, accurate, and precise HPLC method was validated for the determination of sparfloxacin in human serum. It uses a simple protein precipitation with dilution and chromatography on a Luna column. The present method was successfully applied to a pharmacokinetic study of sparfloxacin in human volunteers and has been used in broader-population pharmacokinetic studies of sparfloxacin, which will be presented elsewhere. The relationship between each SNP or haplotype of the *MDR1* genes and the sparfloxacin pharmacokinetic parameters (AUC and C_{max}) did not show any statistically significant differences ($P < 0.05$).

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