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Clinical Pharmacokinetic and Bioequivalence Studies of Two Brands of Cephradine in Healthy Korean Using HPLC Method

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Abstract

The goal of our research was to compare the pharmacokinetics and evaluate the bioequivalence of two brands of cephradine 500 mg capsules in 24 normal Korean volunteers. The plasma samples were acquired at 13 time points for 8 h after administration. The concentrations of cephradine in human plasma were measured by a high-performance liquid chromatography (HPLC). Isocratic mobile phase which consisted of acetonitrile, methanol, and 20 mM potassium phosphate (15/5/80, v/v/v, pH 3.48) was used to separate the analytical column cosmosil cholester (250 × 4.6 mm, 3 μm). Analytes were detected in ultraviolet (260 nm). The novel analytical method was described as simple sample preparation, a short retention time (less than 6 min) and making it suitable for use in clinical trials. Pharmacokinetic parameters, such as $AUC_{0\text{-}t}$ (20.54 vs 18.42 μg·h/mL), $AUC_{0\text{-infinity}}$ (21.22 vs 19.14 μg·h/mL), C_{max} (12.69 vs 12.81 μg/mL), T_{max} (1.22 vs 0.92 h), half-life (1.02 vs 1.13 h), extrapolation (3.22% vs 3.75%), and Ke (0.73 vs 0.69 h^{-1}) were determined for the reference and test drugs in plasma. Pharmacokinetic parameters with a 90% confidence interval were 87% - 95% for $AUC_{0\text{-}t}$ and 91% - 115% for C_{max}. They were satisfied within the bioequivalence range 80% - 125% of the KFDA guidelines. Therefore, our HPLC method was well applied in a bioequivalence and pharmacokinetic study of two formulations in normal subjects.

Keywords

Bioequivalence, Cephalosporin Antibiotic, Cephradine, Korean Volunteers, Pharmacokinetics

1. Introduction

Cephradine ((6R, 7R)-7-{[(2R)-2-amino-2-cyclohexa-1, 4-dien-1-ylacetyl] ami-

no}-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid) is a first generation cephalosporin antibiotic. It has broad spectrum antibacterial activity against gram-positive and gram-negative microorganisms and acts through inhibiting bacterial cell wall synthesis. Cephradine is used to treat upper respiratory infections, ear infections, skin infections, and urinary tract infections. It is rapidly absorbed from the gastrointestinal tract and has low plasma protein binding (6% - 20%) [1]. It is excreted approximately 90% of the drug which is unchanged by the kidney within 6 h administration [2]. A daily therapeutic dose of cephradine is 250 - 500 mg, and after oral administration of 500 mg of cephradine the maximal plasma concentration (C_{max}) is 15.67 ± 4.21 μg/mL and the time to maximal plasma concentration (T_{max}) is about 1 h [3].

Several analytical methods have been reported to measure cephradine in biological fluids. These methods include spectrophotometry [4] [5] [6], spectrofluorimetry [7] [8], luminescence [9] [10], capillary electrophoresis [11], and bioassays [1] [2] [12] [13] [14]. The previous methods often lack sufficient sensitivity and specificity to measure low cephradine concentrations in plasma. Recent studies have used high-performance liquid chromatography (HPLC) [15]-[22] and liquid chromatography tandem mass spectrometry (LC-MS/MS) [23] [24] in order to increase the sensitivity to detect cephradine in biological materials. However, these methods have disadvantages as follows. HPLC methods take a long time and LC-MS/MS methods require the startup costs and highly skilled human resource to perform the assays and manage the instrument. The comparative details of literature are provided in **Table 1**.

Since the Drug Price Competition and Patent Term Restoration Act were implemented in 1984, the US Food and Drug Administration (FDA) have established bioavailability and bioequivalence requirements for generic substitution [13] [25] [26]. According to the Act, the goal of clinical bioavailability studies is to compare certain pharmacokinetic (PK) parameters of the test and reference products. The current bioequivalence test requires, to declare bioequivalence, the population mean differences in the extent (area under the curve (AUC)) and rate of absorption (C_{max}) between the test and reference formulations do not exceed 20% [25]. Therefore, our purpose of research was to develop a fast, convenient, and efficient method to analyze biological cephradine levels and use it to measure cephradine concentrations in human plasma samples in the bioequivalence and pharmacokinetic test of 2 brands of cephradine capsules (500 mg) in 24 normal Korean subjects.

Furthermore, we performed a comparison of basic PK parameters such as AUC, C_{max}, T_{max} and $t_{1/2}$ of cephradine for Korean subjects and the other races, since previous PK studies [1] [13] [22] [23] [27] of cephradine after oral administration did not considered variable ethnic groups .

2. Materials and Methods

2.1. Chemical and Reagents

Cephradine and ofloxacin (Internal Standard (IS)) were purchased from Sig-

ma-Aldrich Co. (St. Louis, Mo, USA). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Philipsburg, NJ, USA). Water was purified using a Milli-Q purification system (Millipore Co., MA, USA). All other chemicals and solvents were HPLC-analytical grade. The reference drug was Yuhan Cephradine capsules 500 mg (Yuhan Pharm Co. Ltd. Seoul, Korea), and the test drug was Korus cephradine capsules 500 mg (Hankook Korus Pharm Co. Ltd. Seoul, Korea).

2.2. Instrumentation and Analytical Conditions

The HPLC system consisted of an isocratic solvent delivery pump (Model 510 pump, Waters Scientific Co., USA), an autosampler (Model 717 Plus, Waters Scientific Co.) an analytical column cosmosil cholester (250 × 4.6 mm, 3 μm) (Nacalai, Kyoto, Japan) and a variable wavelength ultraviolet detector (Model 486 Tunable Absorbance Detector, Waters Scientific Co.) set at 260 nm. Data was acquired and processed with Empower 3.0 software. The mobile phase consisted of acetonitrile, methanol, and 20 mM potassium phosphate (15/5/80, v/v/v, pH 3.48). The flow rate of the mobile phase was 1.3 mL/min and a sample volume of 50 μL was injected into the chromatography system.

2.3. Preparation of Standards and Plasma Samples

Primary stock solutions of cephradine and IS were prepared in 50% aqueous methanol (1:1 methanol/water, v/v) to final concentrations of 1 mg/mL and 200 μg/mL, respectively, and both were kept at −70˚C. A set of seven non-zero calibration standards, ranging from 0.3 ~ 50 μg/mL, was prepared for blank human plasma with an appropriate amount of cephradine. The quality control (QC) samples were prepared in blank plasma at cephradine concentrations of 0.3 (Lower Limit of Quantification (LLOQ)), 1 (low-middle), 10 (high-middle) and 50 μg/mL (high). Blank plasma was tested before spiking, to ensure that there was no endogenous interference before measuring the retention times of cephradine and IS. Frozen human plasma was thawed at room temperature and centrifuged at 3000 rpm for 10 min at 4˚C to precipitate the sediment. A 500-μL aliquot of plasma was transferred to a screw cap glass tube with 50 μL of IS working solution (IS, 200 μg/mL) and 100 μL of acetonitrile/perchloric acid (1:1, v/v). The mixture was vortexed for 15 sec. After centrifugation at 12,000 rpm for 5 min, the supernatant was transferred to a 2 ml tube and centrifuged again. Following centrifugation, the supernatant was transferred to an autosampler vial and an aliquot (50 μL) was injected into the HPLC system.

2.4. Assay Validation

The assay was validated according to the FDA guidance on validation of bioanalytical methods [28] [29]. Linearity was determined with a linear least-squares regression with $1/x^2$ weighting, performed on the peak area ratios of cephradine and IS versus the cephradine concentrations of the seven blank plasma stan-

dards. They were randomly selected under controlled conditions and performed of the same extraction method. The sensitivity of the method was expressed as the LLOQ which could be determined with ideal accuracy and precision. The intra- and inter-day accuracy and precision were evaluated by analyzing QC samples which are four cephradine concentrations (0.3, 1.0, 10, and 50 μg/mL) in plasma from five replicates on the same day and five consecutive days. Precision was expressed as percent coefficient of variation (% CV). Accuracy was showed as the percent ratio between the experimental and nominal concentration for each sample. The LLOQ was defined as the lowest concentration of cephradine standard sample, which both the precision and accuracy were within 20% [28].

2.5. Bioequivalence and Pharmacokinetic Test in Normal Subjects

To evaluate the applicability of this method, a randomized, two-period, and crossover design was used to study the pharmacokinetics and bioequivalence of two types of cephradine in 24 normal Korean subjects.

The study was performed according to the World Medical Association Declaration of Helsinki (1997) for biomedical research involving human subjects in 2007 [30] and protocol was confirmed by the Institutional Review Board of Hanyang University Medical Center [31]-[38]. This study design and number of subjects were properly decided to achieve sufficient statistical power to demonstrate BE test according to the Korean FDA Guidelines [39]. The candidate subjects were recruited by internet and paper notice. Enrolling subjects were selected to depend on inclusion criteria of the Korean FDA Guidelines [39]. Twenty-four subjects aged between 19 - 25 years (21.8 ± 2.4 years), with heights between 169 - 180 cm (174.0 ± 3.6 cm) and body weights between 58 - 83 kg (67.3 ± 6.4 kg) were selected. The subjects were non-alcoholics and disease-free and were assessed as being healthy by a clinical evaluation, including a physical examination and routine clinical laboratory tests including a pregnancy test. One capsule of cephradine (500 mg) was given randomly to 24 healthy subjects. Participants had not taken other medication (including OTC regimens) for two weeks before the study, and refrained from taking other medication during the study period, including the washout period. During each study period, the volunteers were hospitalized at Hanyang University Medical Center at 5:00 pm. and had supper before 8:00 pm. After an overnight fast, they were administered with a test or reference formulation along with 240 mL water at 7:30 am. Food and beverage were not permitted until 4 h after dose. A regular lunch was provided at 4 h after dose and the vital signs of subjects were recorded at times before and after dose. Blood Samples (9 mL) were collected through a catheter with heparin-containing tubes from a suitable antecubital vein before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 h after dose. The samples were centrifuged at 3000 rpm for 10 min at room temperature and the plasma was stored at −70˚C until analyzed [34] [35] [36] [37] [38]. The C_{max} and T_{max} were determined from each subject's plasma level of cephradine versus time plots. Other PK parameters were analyzed and calculated to using PK Solutions software as non-compartmental

PK model [40]. The area under the plasma concentration versus time curve ($AUC_{0\text{-}t}$) was obtained by using the linear trapezoidal rule. The area under the plasma concentration versus time curve extrapolated to infinity ($AUC_{0\text{-}\infty}$) was obtained as $AUC_{0\text{-}t} + C_t/Ke$, where C_t was the last determining concentration, and Ke was the elimination rate constant. The terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda$. The first moment versus time curve ($AUMC_{0\text{-}\infty}$) was obtained by integrating the time (t) of first moment ($C_t \cdot t$) ($AUMC_{\infty} = \int_0^t Ct \cdot t \cdot dt + Cp \cdot \frac{t}{\lambda z^2}$). The mean residence time (MRT) of cephradine in the body was calculated as AUMC/AUC, with λ_z being the Ke [40]. To assess bioequivalence of the test and reference drugs, $AUC_{0\text{-}t}$ and C_{max} were considered to be the primary variables, and a 2-way ANOVA for the crossover–randomized design was used to estimate the effect of types, term, sequence, and subjects on these parameters [41]. Differences between 2 related parameters were regarded statistically significant at $p < 0.05$. The 90% confidence intervals (CI) of the geometric means for the individual test/reference ratios (T/R) for $AUC_{0\text{-}t}$, and C_{max} were calculated, in order to evaluate bioequivalence of the 2 types. The parametric 90% CIs for the ratios in the 80% - 125% range suggested by the Korean Food and Drug Administration (KFDA) were calculated using parametric methods for log-transformed data [41].

3. Results and Discussion

3.1. Analysis

The molecular structures of cephradine and ofloxacin (IS) were indicated in **Figure 1**. Samples were prepared by precipitating protein in aqueous perchloric acid/acetonitrile (1:1, v/v) and centrifuging the suspension at 12,000 rpm for 5 min at 4˚C. The supernatants were directly injected into the HPLC system. To separate cephradine and IS from the endogenous plasma matrix, we used a reverse-phase column. This column offered improved separation for compounds such as β-lactam antibiotics that were difficult to analyze with other columns.

The following chromatograms were shown in **Figure 2**: (a) double blank plasma without cephradine and IS, (b) blank plasma with 200 μg/mL of IS, (c) blank plasma spiked with 0.3 μg/mL (LLOQ) of a calibration standard of cephradine and 200 μg/mL of IS, and (d) human plasma taken 1.5 h after a single oral dose of cephradine (500 mg tablet) and spiked with 200.0 μg/mL of IS. The retention times of cephradine and IS were 4.13 min and 5.55 min, respectively, none of the blank plasmas yielded any significant endogenous peaks in the cephradine or IS retention times (**Figure 2(a)**). Our HPLC method was showed short run time (7 min) than previous methods (10 - 28 min) [15]-[22] (**Table 1**).

3.2. Method Validation and Linearity of Calibration

A calibration curve was constructed from the peak area ratios of cephradine against IS by using a double-blank sample (plasma sample without cephradine

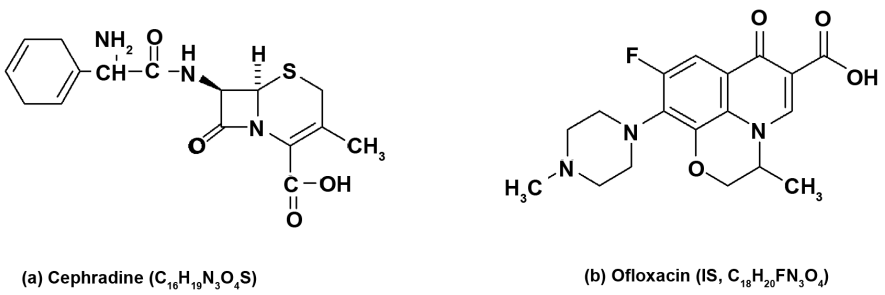

Figure 1. Chemical structures of (a) Cephradine and (b) Ofloxacin (internal standard).

or IS) and seven calibration standard concentrations (0.3 ~ 50 μg/mL). The standard calibration curve was indicated good linearity within the range of 0.3 - 50 μg/mL by least-squares regression analysis (y = 0.0521, x − 0.0022, r^2 = 0.9999, $1/x^2$ weighting). Intra- and inter-day precision and accuracy were determined by analyzing QC samples against the calibration curve, on the same day (n = 5) and on different days (n = 5). As indicated in Table 2, intra- and inter-day precisions were 3.27% - 4.75% and 4.56% - 6.22%, respectively, and intra- and inter-day accuracies were 99.71% - 105.65 % and 97.48% - 99.86 %, respectively. The LLOQ was decided to accept the 0.3 μg/mL under the analytical conditions of the precision and accuracy less than 20% [28]. Therefore, our research was yielded sufficiently the analytical method for pharmacokinetics and bioequivalence studies.

3.3. Pharmacokinetics and Bioequivalence

The proposed method was applied to the determination of cephradine in plasma samples for the purpose of establishing the pharmacokinetic and bioequivalence study of 500 mg cephradine formulations in 24 healthy Korean volunteers [42]. The demographic characteristics for each of the healthy volunteers studied are given in Table 3. There were 22 men and 2 women. Subjects were randomly assigned to receive the reference or test formulation.

The PK parameters for the reference and test drug obtained were described as follows. The first sampling time of 24 subjects were measurable at 0.25 h after oral administration of cephradine. The profiles of the plasma cephradine concentration vs time were shown in Figure 3. Even though the bioavailability of orally administered cephradine is characterized by considerable individual variation, plasma concentration-time profiles of the two formulations were similar patterns.

The PK parameters of cephradine were shown in Table 4 and Table 5. In previous studies [2] [13] [23] [27] (Table 5), the PK data of cephradine after oral administration were rapid with 0.80 - 1.25 h of T_{max}, 0.61 - 0.95 h of half-life ($T_{1/2}$), 12.11 - 17.7 μg/mL of C_{max}, and 23.14 - 27.52 μg·h/mL of AUC_{∞} under similar conditions. The mean T_{max} values under different conditions of cephradine 1 kg and 250 mg were 1.12 ± 0.24 h and 0.76 ± 0.12 h, respectively [2] [22]. Our results agreed well with these parameter values. Therefore, it means that there were no difference between Korean subject and the other races.

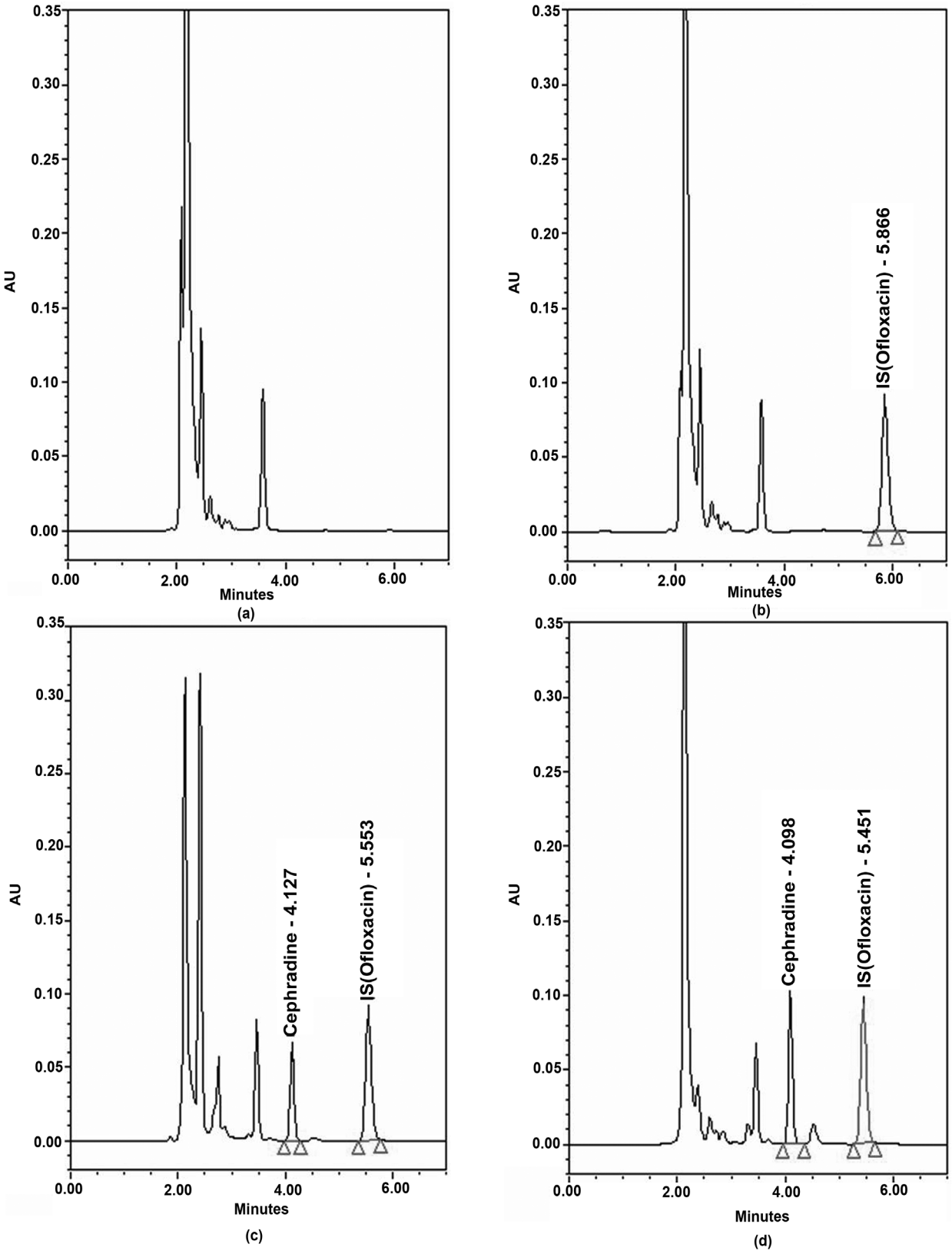

Figure 2. Chromatograms of (a) Double-blank plasma without cephradine or IS, (b) Blank plasma with IS (200 μg/mL), (c) Blank plasma spiked with cephradine (LLOQ, 0.3 μg/mL) and IS (200 μg/mL), and (d) A subject's plasma taken 1.5 h after a single oral cephradine 500 mg capsule and spiked with IS (200.0 μg/mL).

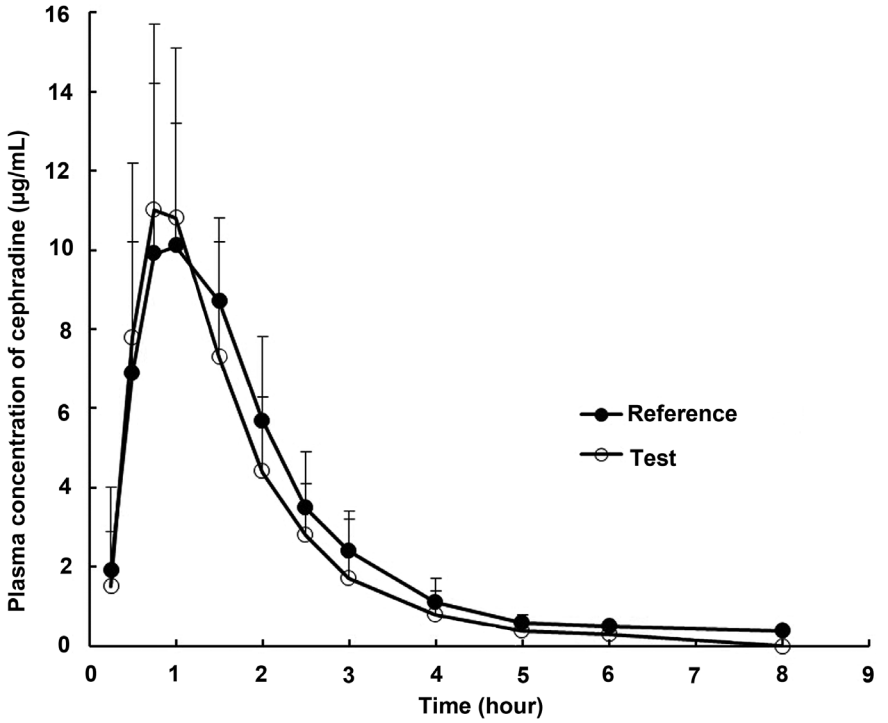

Figure 3. Mean plasma concentrations versus time after single oral cephradine 500 mg capsules were given to 24 healthy subjects (●: reference, ○: test) (n = 24, mean ± SD).

Table 1. Comparison of HPLC methods from articles.

Applied Method	LOD	Linearity	Run times	Sample type
Cho HY *et al.* 2002 [3]	0.2 μg/mL	0.2 - 30.0 μg/mL	10 min	Human plasma
Johnson VM e*t al.* 2000 [15]	0.2 μg/mL	0.2 - 30.0 μg/mL	18 min	Human plasma
Shoaib MH *et al.* 2008 [22]	0.4 μg/mL	1 - 12 μg/mL	28 min	Human plasma

Table 2. Validation of the method for analyzing cephradine in human plasma (n = 5).

Nominal Concentration	Precision (%CV)		Accuracy (%)	
(μg/mL)	Intra-day	Inter-day	Intra-day	Inter-day
0.3 (LLOQ)	4.15	6.22	99.71	97.48
1 (low)	3.71	4.83	105.53	99.07
10 (middle)	4.75	5.43	105.65	99.86
50 (high)	3.27	4.56	103.01	97.86

LLOQ = lower limit of quantification, CV = coefficient of variation.

In order of the reference and test formulation of cephradine, C_{max} were 12.69 and 12.81 μg/mL and T_{max} were 1.22 and 0.92 h, respectively. The mean $AUC_{0\text{-}t}$ values were 20.54 and 18.42 μg·h/mL and the mean $AUC_{0\text{-}\infty}$ values were 21.22 and 19.14 μg·h/mL. In addition, the other PK parameters of two formulations of 500 mg cephradine after oral administration were as follows. The mean $AUMC_t$

Table 3. Demographic Characteristics and sequence of drug administration of the 24 healthy volunteers that participated in the study. (R: Reference drug, T: Test drug).

Subject	Gender (M/F)	Age (years)	Height (cm)	Weight (kg)	Sequence
1	M	24	173.1	65.6	T-R
2	M	26	175	50.6	T-R
3	M	26	174.5	66.3	T-R
4	M	33	173.3	55.2	T-R
5	F	30	157.1	47	R-T
6	M	23	175	62	R-T
7	M	26	171.7	60.4	R-T
8	M	19	178.8	84	R-T
9	M	25	176	66	R-T
10	M	27	181.7	69	T-R
11	F	35	159.3	55.8	R-T
12	M	25	176	92	R-T
13	M	24	176	74	R-T
14	M	23	173	75	R-T
15	M	25	168.6	61	T-R
16	M	23	170.2	61.5	T-R
17	M	24	174.5	68.5	T-R
18	M	24	179.7	79.3	T-R
19	M	27	176.6	78.6	R-T
20	M	29	169.7	62.1	T-R
21	M	24	177.5	87.5	T-R
22	M	25	177.9	75.8	R-T
23	M	35	167.9	68.7	R-T
24	M	28	167.5	72.7	T-R

values were 39.34 and 28.56 μg·h^2/mL, and $AUMC_{\infty}$ values were 39.93 and 33.47 μg·h^2/mL for the reference and test drug. The mean MRT value obtained for the reference and test drug were 1.86 and 1.73 h, respectively. These parameters almost overlapped between the test and reference drugs (Table 4).

The 90 % CI of the test/reference percentage ratio were 90.6% (86.64% - 97.74%) for $AUC_{0\text{-}t}$ and 102.3% (91.06% - 114.84%) for C_{max}, both of which were within the bioequivalence limits of 80% - 125% for the percentage ratio of product averages. These results support that the two branded formulations of 500 mg cephradine capsules were bioequivalent. Since we evaluated the minimum number of healthy adult subjects, this study has no consideration for all ages and gender.

Table 4. Pharmacokinetic parameters (mean ± SD) of two formulations of cephradine 500 mg capsules based on plasma concentrations in 24 healthy subjects.

Parameters	Reference drug	Test drug
AUC_t (μg·h/mL)	20.54 ± 4.30	18.42 ± 2.96
AUC_∞ (μg·h/mL)	21.22 ± 4.38	19.14 ± 3.06
Extrapolated AUC (%)	3.22 ± 1.05	3.75 ± 1.73
$AUMC_t$ (μg·h^2/mL)	35.34 ± 13.56	28.56 ± 7.88
$AUMC_\infty$ (μg·h^2/mL)	39.93 ± 15.37	33.47 ± 9.19
Vd (L)	36.68 ± 11.25	45.18 ± 20.68
MRT (h)	1.86 ± 0.39	1.73 ± 0.29
C_{max} (μg/mL)	12.69 ± 3.27	12.81 ± 2.80
$T_{1/2\alpha}$ (h)	0.29 ± 0.15	0.40 ± 0.27
T_{max} (h)	1.22 ± 0.57	0.92 ± 0.32
k_a (h^{-1})	3.23 ± 2.64	2.46 ± 1.58
$T_{1/2\beta}$ (h)	1.02 ± 0.31	1.13 ± 0.57
λ_z (k_e, h^{-1})	0.73 ± 0.17	0.69 ± 0.19
CL (L/h)	25.36 ± 5.26	27.83 ± 4.57

AUC_t = area under the plasma concentration-time curve from time 0 to time *t*, AUC_∞ = area under the plasma concentration-time curve from time 0 to infinite time, $AUMC_t$ = area under the first moment of the plasma concentration-time curve from time 0 to time *t*, $AUMC_\infty$ = area under the first moment of the plasma concentration-time curve from time 0 to infinite time, Vd = the apparent volume of distribution, MRT = the mean residence time, C_{max}= the maximal plasma concentration, $T_{1/2\alpha}$ = the half-life of absorption, T_{max} = the time to maximal plasma concentration, k_a = the distribution rate constant, $T_{1/2\beta}$ = the half-life of elimination, λ_z(k_e) = the elimination rate constant, CL= clearance.

Table 5. Comparison of Pharmacokinetic parameters (mean ± SD) of cephradine after oral administration in current and previous studies.

Parameters / Precious reports	Country	Sample Size	Sampling Time	AUC_t (μg·h/mL)	AUC_∞ (μg·h/mL)	C_{max} (μg/mL)	T_{max} (h)	$T_{1/2\beta}$ (h)
Current study	Korea	24	8 h	18.42 ± 3.0	19.14 ± 3.1	12.81 ± 2.8	0.92 ± 0.3	1.13 ± 0.6
Hassanzadeh MK *et al.* 1999 [1]	Iran	8	6 h	-	23.14 ± 2.3	12.11 ± 2.7	1.25 ± 0.4	0.95 ± 0.2
Chen ML 1992 [13]	USA	18	10 h	24.7 ± 2.7	25.1 ± 2.7	17.4 ± 0.6	0.83 ± 0.1	-
Shoaib, MH 2008 [22]	Pakistan	6	7 h	16.40 ± 1.7	16.90 ± 0.3	11.49 ± 1.7	0.76 ± 0.1	0.44 ± 0.2
Choi SJ *et al.* 2009 [23]	Korea	24	6 h	26.99 ± 4.2	27.52 ± 4.2	16.3 ± 4.3	1.2 ± 0.5	0.85 ± 0.1
Pfeffer M *et al.* 1977 [27]	USA	12	12 h	-	27.5 ± 2.2	17.7 ± 1.2	0.80 ± 0.1	0.61 ± 0.1

4. Conclusion

We developed a fast HPLC method to measure cephradine in human plasma. It was based on removing protein by precipitation, and used a high resolution column and a fast isocratic flow rate. This method had similar precision and accuracy to previous methods, whereas, it had indicated more short analysis times (cephradine [4.13 min], IS [5.55 min]) than previous analysis methods. The use of a method with a short retention time was important, particularly for large-scale clinical trials, because it allowed its high throughput. The method was

successful in a strictly-controlled bioequivalence and pharmacokinetic study of two brands of cephradine 500 mg capsules in 24 normal subjects.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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