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A specific and rapid HPLC assay for the determination of cefroxadine in human plasma and its application to pharmacokinetic study in Korean

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Abstract

A specific and rapid high performance liquid chromatographic (HPLC) method with UV detection (254 nm) was developed for the determination of cefroxadine in human plasma. The sample extraction was performed by a simple procedure, vortexing and centrifugation of sample following addition of 60% trichloroacetic acid. Cephalexin was used as an internal standard (I.S.). The HPLC analysis was carried out on a Capcell Pak C_{18} analytical column with a mobile phase of 50 mM ammonium formate buffer/pH 3.5 and acetonitrile (90:10, v/v). No interference was observed near the peaks of cefroxadine and I.S. The calibration curve was linear over the range of 0.5–40 μg/mL and the lower limit of quantification (LLOQ) was 0.5 μg/mL. The method was validated with excellent sensitivity, accuracy, precision and stability. This assay was successfully applied to determine the pharmacokinetic parameters of cefroxadine in Korean healthy volunteers after an oral administration of two 250 mg cefroxadine capsules. As a result, the plasma half-life was 1.00 ± 0.26 h and the mean $AUC_{0-6\,h}$ was 46.25 ± 6.41 μg h/mL. The maximum plasma concentration (C_{max}) of 17.62 ± 4.87 μg/mL reached 1.44 ± 0.39 h after administration.

Keywords: Cefroxadine; HPLC; Human plasma; Pharmacokinetics; Validation

1. Introduction

Cefroxadine (7β-[D-2-amino-2-(1,4-cyclohexadienyl)-acetamido]-3-methoxy-ceph-3-em-4-carboxylic acid, Fig. 1A) is one of the frequently prescribed oral cephalosporins with a broad antibacterial spectrum [1–3]. For the determination of the pharmacokinetic parameters of cefroxadine in biological samples, microbial assays or high pressure liquid chromatography (HPLC) has been used in a number of studies [4–11]. Most of the HPLC methods reported previously are mainly based on the method developed by Lecaillon et al. [5,8,9,11,12]. Although the HPLC method was more accurate and precise than microbiological determination, the peaks were not well resolved and had slow retention times. The HPLC method reported by Bergan and Solberg [13] had also some problems despite the rapid retention time. In the study, cefroxadine was assayed without internal standard (I.S.) and the peak was not well separated from the peaks of endogenous plasma compounds. Furthermore, the method was not properly validated, and the appropriate method for the determination of cefroxadine in human plasma has not been recently reported. Therefore, the objective of this study was to develop and validate a rapid and specific HPLC method for the quantification of cefroxadine in human plasma. Also, by applying this method to the analysis of the plasma sample of Korean

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healthy volunteers following oral dosing of cefroxadine, we examined the pharmacokinetic parameters of cefroxadine in Korean subjects for the first time.

2. Experimental

2.1. Chemicals

Cephalexin was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate, formic acid and trichloroacetic acid were supplied by Junsei Chemical Co. Ltd. (Tokyo, Japan). Cefroxadine and Tiroxin® capsules (250 mg cefroxadine) were obtained from Samjin Pharm. (Seoul, Korea). All the chemicals were analytical grade.

2.2. Preparation of stock and standard solutions

Cephalexin (7-(D-α-amino-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid, Fig. 1B) is structurally associated with cefroxadine and can be determined simultaneously by HPLC with similar retention time to and good separation from the cefroxadine [8], therefore, cephalexin was chosen as I.S. in this study. Stock solutions of cefroxadine and cephalexin (I.S.) were prepared in distilled water at concentrations of 200 and 100 μg/mL, respectively. The stock solutions were stored at 4 °C until analysis. Standard solutions were prepared by diluting the cefroxadine stock solution with distilled water, giving final concentrations of 5, 10, 20, 50, 100, 200 and 400 μg/mL.

2.3. Chromatographic conditions

All the analyses were performed using the Waters HPLC system (Milford, MA, USA) consisted of a Model 510 pump, a Model 486 tunable absorbance detector, a Model 715 ultra wisp sample processor and ERIC-3215 degasser. The chromatographic separation was carried out with a Capcell Pak C_{18} analytical column (5 μm particle size, 250 mm × 4.6 mm, Shiseido, Tokyo, Japan). The analytical column was protected by an Ultra C_{18} guard cartridge (5 μm particle size, 4.0 mm × 10 mm, Restek, Bellefonte, PA, USA). The mobile phase, consisting of 50 mM of ammonium formate buffer and acetonitrile (90:10, v/v), was run at a flow rate of 1.2 mL/min, and the buffer pH was adjusted to 3.5 with formic acid. The 50 μL aliquots of samples were injected onto the column and the eluates were monitored with UV detector at 254 nm. All the procedures were performed at room temperature.

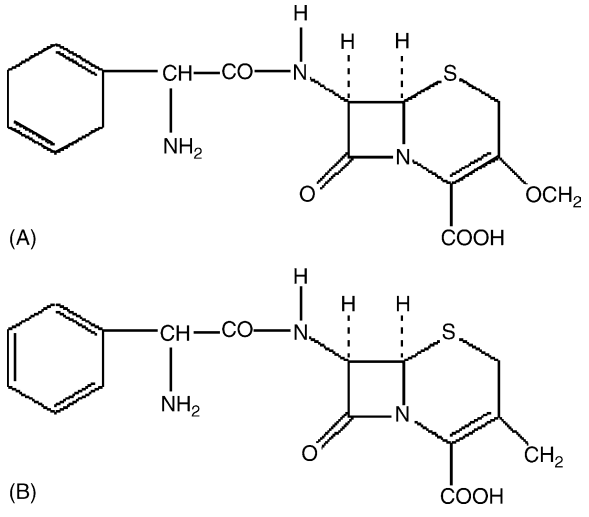

Fig. 1. Chemical structures of cefroxadine (A) and cephalexin (I.S., B).

2.4. Preparation of samples

The sample preparation method reported by Lecaillon et al. [5] was modified in this study. To 200 μL of plasma, 10 μL of internal standard solution and 20 μL of 60% trichloroacetic acid were added. After shaking for one minute, the samples were centrifuged at 14,000 rpm for 10 min. A 50 μL aliquot of supernatant was injected onto the HPLC column.

2.5. Validation

2.5.1. Specificity

The specificity of the method was verified by investigating the peak interference from the endogenous plasma substances. The chromatogram of the human plasma spiked with cefroxadine and I.S. was compared to that of the blank plasma sample.

2.5.2. Linearity

The linearity of the method was evaluated by a calibration curve in the range of 0.5–40 μg/mL of the drug ($n = 5$). Drug-free plasma was spiked with cefroxadine standard solutions to achieve final concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 40.0 μg/mL. Calibration curve was obtained by plotting peak area ratios of cefroxadine to I.S. versus the cefroxadine concentrations with least-squares linear regression analysis.

2.5.3. Precision and accuracy

Intra-day and inter-day precision and accuracy were determined by replicate analysis of five sets of samples spiked with four different concentrations of cefroxadine (0.5, 1.0, 5.0 and 20.0 μg/mL) within a day or during five consecutive days. The precision was calculated from the ratio of the standard deviation to the mean (coefficient of variation, CV). The accuracy of the method was examined by comparing the concentrations of spiked samples to the theoretical concentrations. Both values were expressed as percentage. The acceptable range of intra-day and inter-day accuracy and precision are below 15% bias or CV.

2.5.4. Sensitivity

The sensitivity was evaluated by the lower limit of quantification (LLOQ), the lowest concentration of the plasma spiked with cefroxadine in the calibration curve. The LLOQ was defined as the concentration producing a precision less than 20% and accuracy between 80% and 120% of the theoretical concentrations.

2.5.5. Recovery

The recovery was determined by comparing peak area of cefroxadine after extraction to that before extraction at concentrations of 5.0, 10 and 20.0 μg/mL.

2.5.6. Stability

Standard stock solution stabilities at the concentration of 1.0 and 10.0 μg/mL of cefroxadine and of 5 μg/mL of I.S. were obtained by analyzing samples left at 4 °C for 6 h. Freeze-thaw stability of the plasma samples was determined by the following three freeze-thaw cycles. The spiked plasma samples at concentrations of 1.0 and 10.0 μg/mL were frozen at −70 °C for 24 h and thawed at room temperature. After completely thawed, the samples were refrozen and this cycle was repeated three times. For the short-term stability, plasma samples were kept at room temperature for 4 and 8 h before the sample preparation. The long-term stability was evaluated after freezing the plasma samples at −70 °C for 3 weeks. The stability of the prepared plasma samples at the concentration of 2.0 μg/mL was tested after keeping the samples for 6 and 12 h at room temperature. The stabilities were determined by the difference of measured sample concentration from the concentration of sample at 0 h.

2.6. Pharmacokinetic study of cefroxadine in Korean subjects

The validated method was applied to pharmacokinetic study of cefroxadine in nine healthy Korean volunteers (aged between 19 and 26 years (23.78 ± 2.11), weighing between 62 and 75 kg (70.67 ± 7.27)) after an oral administration of 500 mg cefroxadine (two Tiroxin® capsules, 250 mg/capsule). The volunteers were fasted overnight before the study and for 4 h after the dosing. Venous blood samples (5 mL) were collected at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, 3, 4, 5 and 6 h following administration. The blood samples were centrifuged at 3500 rpm for 6 min and the plasma was taken and stored at −70 °C until analysis. Pharmacokinetic parameters were evaluated from plasma concentration–time curve using WinNonlin software (Pharsight Co., Mountain View, CA, USA) with non-compartmental analysis. The pharmacokinetic parameters estimated from the data were as follows; the area under the plasma concentration–time curve (AUC), the maximum plasma concentration (C_{max}), the time required to reach C_{max} (T_{max}), elimination half-life ($t_{1/2}$) and elimination rate constant (Ke). This study was approved by the institutional review board at the Research Institute of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, after having received written consents from all the volunteers.

3. Results and discussion

3.1. Chromatography

As shown in Fig. 2B, the chromatogram showed a clear and good separation, and the retention times of cefroxadine and I.S. were approximately 7.1 and 8.1 min, respectively. Based on our results, the retention time of cefroxadine was faster than that reported by Lecaillon et al. [12] and complete separation between cefroxadine and endogenous interferences was obtained, which was not fully achieved by Bergan and Solberg [13].

3.2. Specificity

As mentioned above, under the described analysis procedure, the peaks of cefroxadine and I.S. were well resolved with good symmetry and desirable retention time from endogenous compounds in the blank human plasma. Representative chromatograms of human blank plasma and plasma samples spiked with cefroxadine and I.S. were shown in Fig. 2. There were no interference peaks near the retention times of cefroxadine and I.S.

3.3. Linearity

The calibration curve was linear over the cefroxadine concentration range from 0.5 to 40 μg/mL in human plasma. The calibration equation from five replicate experiments, $y = 0.0511x - 0.0023$ ($r^2 = 0.9999$, $p < 0.01$), demonstrated the linearity of the method.

3.4. Precision and accuracy

The results of precision and accuracy were presented in Table 1. The intra-day precision and accuracy were varied between 1.7 and 6.3%, and 89.7 and 105.3%, respectively. The inter-day precision and accuracy ranged from 3.9 to 10.0% and 89.4 to 104.8%, respectively. All the values of precision and accuracy including LLOQ were within the specified ranges and therefore acceptable.

Table 1
Intra- and inter-day precision and accuracy of cefroxadine spiked in human plasma ($n = 5$)

Concentration (μg/mL)	Precision (CV, %)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.5	6.3	6.2	104.5	104.8
1.0	6.2	10.0	105.3	100.0
5.0	2.6	6.9	101.6	95.2
20.0	1.7	3.9	89.7	89.4

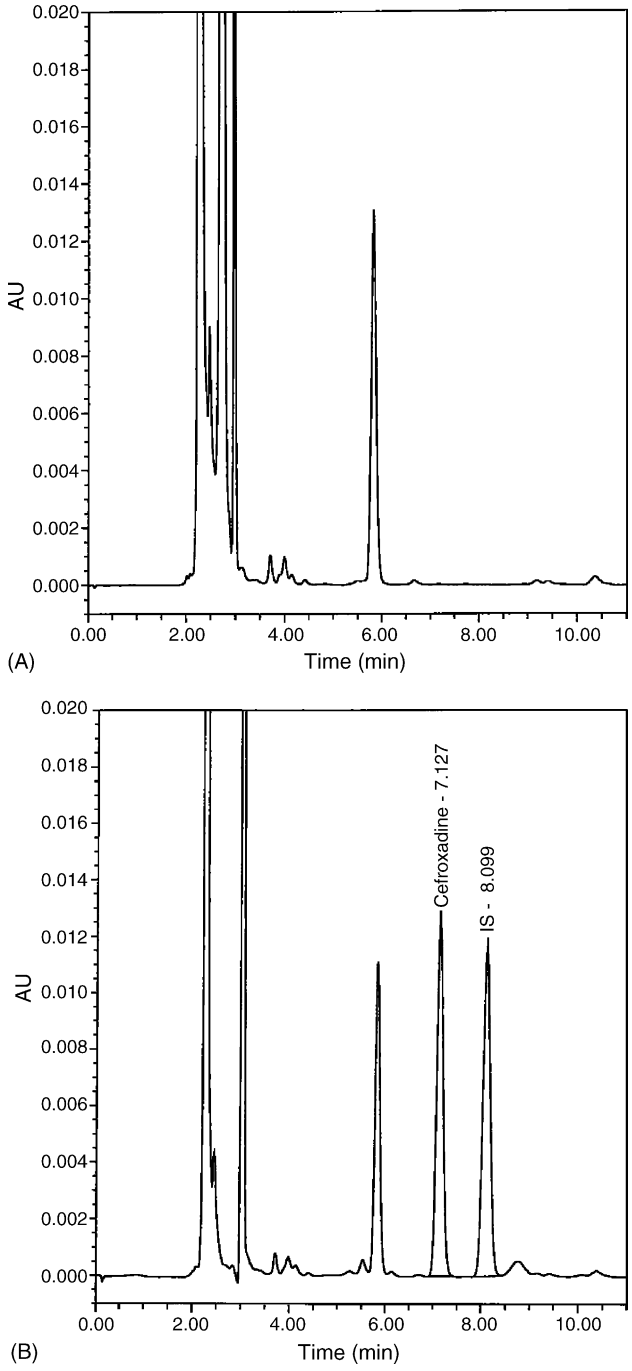

Fig. 2. Representative chromatograms of human blank plasma (A) and plasma spiked with cefroxadine and internal standard (B, cefroxadine: 7.127 min, I.S.: 8.099 min).

3.5. Sensitivity

The LLOQ was determined to be 0.5 μg/mL. The intra-day precision and accuracy were 6.3% and 104.5%, respectively. The inter-day precision and accuracy were 6.2% and 104.8%, respectively.

3.6. Recovery

The mean recovery for the cefroxadine in human plasma was evaluated by triplicate analysis as 110.6 ± 6.8% at 5 μg/mL, 95.16 ± 7.8% at 10 μg/mL and 97.21 ± 1.02% at 20 μg/mL. These results suggest approximately 100% recovery from the preparation procedure of the samples.

3.7. Stability

The stabilities of drug and I.S. in a biological fluid are affected by the chemical properties of drug and I.S, the storage conditions, the matrix and the container systems. The stability of standard stock solution of I.S. was 100.1%. Also, the stability of the prepared plasma samples was 100.5% at 6 h and 100.1% at 12 h. Other results of stability experiments were shown in Table 2. Cefroxadine was stable during sample collection, handling and analysis. Moreover, the stability of cefroxadine was confirmed after the three freeze-thaw cycles, short-term and long-term storage. The conditions used in the stability tests reflected situations most likely to be encountered during actual sample analysis.

3.8. Pharmacokinetic study of cefroxadine in Korean subjects

The plasma samples from nine Korean volunteers were assayed with the validated method described above. The representative chromatograms of a volunteer plasma at 0 h and 1.25 h following an oral administration of cefroxadine were shown in Fig. 3. The peaks of cefroxadine and I.S. were completely separated from endogenous peaks with similar retention times to those of the samples used for the validation studies. Fig. 4 showed the mean plasma concentration–time curve. The mean values of pharmacokinetic parameters estimated by the computer program WinNonlin with non-compartmental method were shown in Table 3. The elimination half-life of cefroxadine in Korean volunteers was in good agreement with the values reported by Bergan and other groups [4–8,11,14]. Bergan [6] previously reported that after an oral administration of

Table 2
Stability of cefroxadine samples (%)

Concentration of cefroxadine (μg/mL)	Stock solution stability	Freeze-thaw stability	Short-term stability		Long-term stability
			4 h	8 h	
1.0	98.6	104.0	93.5	88.1	96.3
10.0	100.4	99.3	93.1	90.3	101.1

Table 3
Pharmacokinetic parameters of cefroxadine after an oral administration of 500 mg cefroxadine (two Tiroxin® capsules, 250 mg/capsule) to nine Korean healthy volunteers

AUC_{0-6h} (μg h/mL)	$AUC_{0-\infty}$ (μg h/mL)	C_{max} (μg/mL)	T_{max} (h)	Ke (h^{-1})	$t_{1/2}$ (h)
46.25 ± 6.41	48.38 ± 7.17	17.62 ± 4.87	1.44 ± 0.39	0.73 ± 0.18	1.00 ± 0.26

Each value was presented as mean ± S.D. AUC_{0-6h}: area under the plasma concentration–time curve from 0 h to 6 h (the end point of blood sampling); $AUC_{0-\infty}$: area under the plasma concentration–time curve from 0 h to infinity; C_{max}: maximum plasma concentration; T_{max}: time required to reach C_{max}; Ke: elimination rate constant; $t_{1/2}$: elimination half-life.

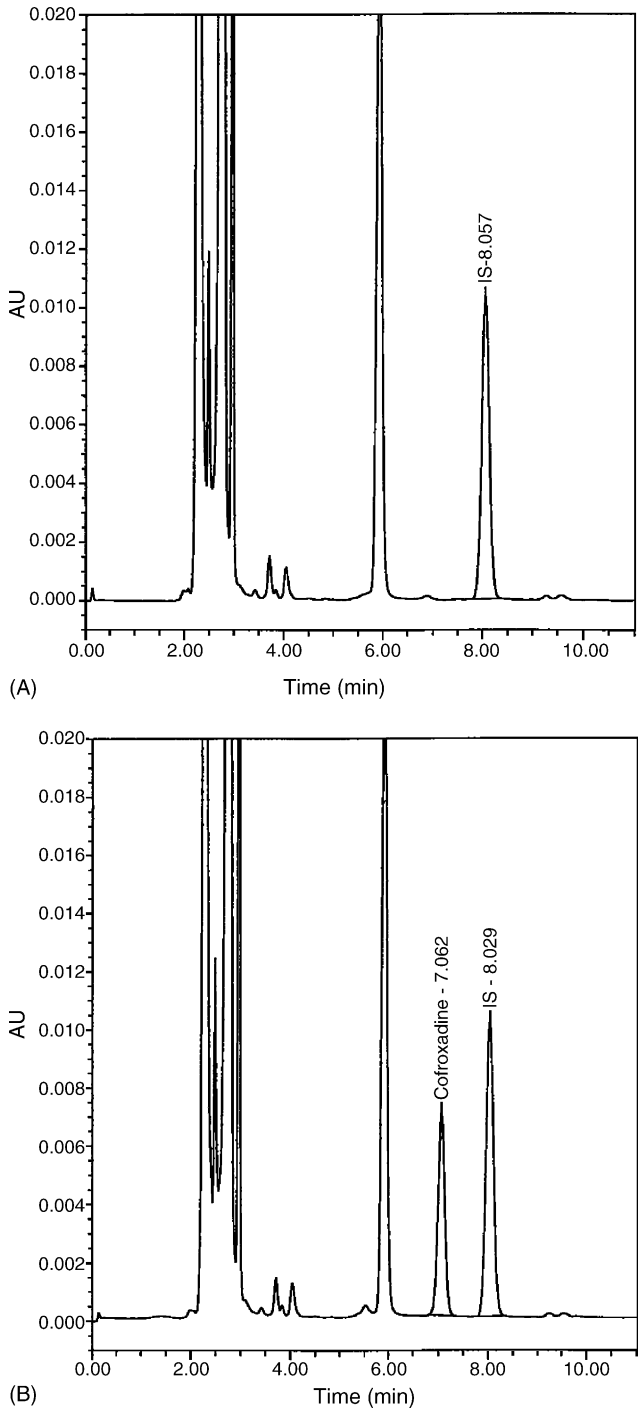

Fig. 3. Representative chromatograms of human volunteer plasma at 0 h (A) and 1.5 h (B) following an oral administration of cefroxadine (cefroxadine: 7.062 min, I.S.: 8.057 (A) and 8.029 (B) min).

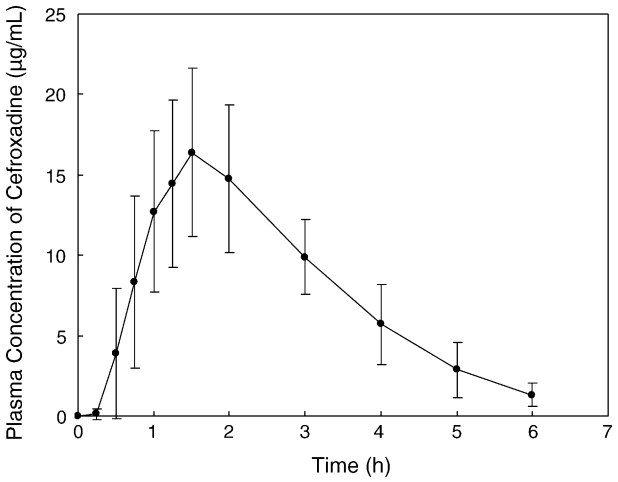

Fig. 4. Mean plasma concentration–time profile after an oral administration of 500 mg cefroxadine (two Tiroxin® capsules, 250 mg/capsule) to nine Korean healthy volunteers.

500 mg cefroxadine to healthy volunteers, AUC, C_{max}, T_{max}, Ke and $t_{1/2}$ were 23.59 ± 2.93 μg h/mL, 12.3 ± 4.2 μg/mL, 0.75 h, 0.864 ± 0.198 h^{-1} and 0.92 ± 0.21 h, respectively. The mean AUC, C_{max} and T_{max} of cefroxadine obtained from Korean healthy subjects (48.38 ± 7.17 μg h/mL, 17.62 ± 4.87 μg/mL and 1.44 ± 0.39 h, respectively) were higher than the reported values by Bergan under the comparable study design [6], at least in part, due to differences in ethnic groups and analytical method (microbiological assay).

4. Conclusion

We validated the HPLC method for simple, rapid, selective and accurate analysis of cefroxadine in human plasma as well as for satisfying the bioanalytical method validation of Korea Food and Drug Administration. Also, by applying this method to analyze the plasma samples of Korean healthy subjects, we determined the pharmacokinetic parameters of cefroxadine in Korean, demonstrating the adequacy of this assay for clinical studies. Furthermore, this analytical method developed in the present study could be used for bioequivalence studies of cefroxadine in the future.

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