Analysis of Ciprofloxacin by a Simple High-Performance Liquid Chromatography Method

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Abstract

A simple and sensitive high-performance liquid chromatographic method is described for the quantitative analysis of ciprofloxacin in pharmaceuticals and human plasma. The method employs reversed-phase chromatography using an RP-C18 column with an isocratic mobile phase of acetonitrile–2% acetic acid aqueous solution (16:84, v/v), umbelliferone as an internal standard, and a flow rate of 1.0 mL/min. The UV detector is set at 280 nm. The limit of detection is 0.25 µM (S/N = 3, injection volume = 10 µL). The regression equations are linear (r > 0.9999) over a range between 0.51~130 µM for the pharmaceutical analysis of ciprofloxacin and 0.51~64.8 µM for the biological analysis of ciprofloxacin in human plasma. The intra- and inter-day relative standard deviation and relative error are less than 3.39% and 5.71%, respectively. All the recoveries are greater than 93.8%. This method is successfully applied in a pharmacokinetic study of a volunteer who receives a 500 mg ciprofloxacin tablet.

Introduction

Ciprofloxacin (Figure 1) is a fluoroquinolone-type antibiotic agent. It exhibits broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria such as *Pseudomonas aeruginosa, Streptococcus faecalis, Staphylococcal aureus*, and *Enterobacter aerogenes* (1,2). It is used in the treatment of a wide range of infectious diseases (3).

There are many reports describing the determination of ciprofloxacin using high-performance liquid chromatography (HPLC) coupled with a UV or fluorescence detector (4–25). However, the current available methods require the use of an acidic mobile phase of three solvents (10,12,20,25), the use of a buffered solution as mobile phase (5,7,8,11,13–15,18,21–23), or the use of an ion-pairing mobile phase (6,9,15,17,23). They may involve an extra-elaborate biological sample preparation such as dichloromethane or solid-phase extraction (5,8,13,14,21,22). It is very time-consuming for an elaborate preparation and thus impractical for the analysis of biological samples of ciprofloxacin.

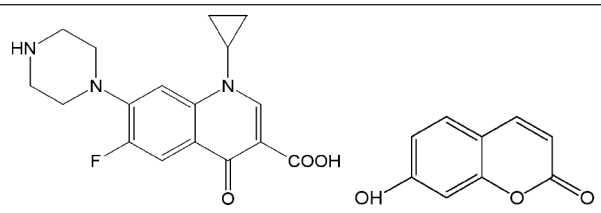

Figure 1. The structures of ciprofloxacin and umbelliferone.

In this paper, we have developed a simple HPLC method using only two solvents, 2% acetic acid aqueous solution and acetonitrile (ACN), with UV detection methodology for accurate determination of ciprofloxacin in human plasma or pharmaceutical preparations without any extraction procedure during sample pretreatment, and without using any buffered solution and ion-pairing reagents as the mobile phase. Furthermore, this method was verified in a pharmacokinetic study of ciprofloxacin using a healthy volunteer who received a single oral dose of ciprofloxacin.

Experimental

Instrument

The HPLC analysis was carried out with a Waters 510 HPLC Pump (Milford, MA), Dynamax AI-3 Automatic Sample Injector (Waltham, MA), Applied Biosystems 785A Programmable Absorbance Detector (Foster City, CA), and Agilent ChemStation software for LC system (Palo Alto, CA).

Reagents

Ciprofloxacin hydrochloride monohydrate was obtained from Chemax (Ahmedabed, India). Umbelliferone was purchased from TCI (Tokyo, Japan) and used as an internal standard for this method. Milli-Q water (Millipore, Bedford, MA) was used for the preparation of the mobile phase and related aqueous solution. ACN was obtained from Tedia (Fairfield, OH). Glacial acetic acid was obtained from Fisher Science (Fairlawn, NJ). A Vacutainer blood collection tube was used to prepare plasma.

Chromatographic conditions

The analyte was separated at ambient temperature using an Alltima C_{18} (4.6 × 150 mm; 5 µm) analytical column (Grace Davison Discovery Sciences, Deerfield, IL) and a Nova-Pak C_{18}

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(4 µm) guard column (Waters). The UV detector was operated at 280 nm. The mobile phase consisted of a mixture of 2% acetic acid aqueous solution and ACN (84:16, v/v). The flow rate was set at 1.0 mL/min and injection volume at 10 µL.

Stock and working solutions

Stock solutions of ciprofloxacin were prepared at 5.18 mM in 2% acetic acid aqueous solution for pharmaceutical and biological analysis, respectively. Umbelliferone was prepared at 6.17 mM in ACN. Solutions were kept at 4°C.

Working solutions of ciprofloxacin were diluted from stock solutions with the mobile phase to 1.01µM, 4.05µM, 16.2µM, 64.8µM, and 259µM for the calibration curve used for pharmaceutical analysis and to 2.53µM, 5.06µM, 20.3µM, 81.0µM, and 324µM for the calibration curve used for biological analysis. Umbelliferone solutions were diluted with ACN to 154µM and 385 µM for pharmaceutical and biological analysis, respectively.

Precision and accuracy

For pharmaceutical analysis, the intra- and inter-day assays in precision and accuracy were executed with five replicates each of low (4.05µM), middle (16.2µM), and high (64.8µM) quality control samples.

For biological analysis, the intra- and inter-day assays in precision and accuracy were executed with six replicates each of low (2.03µM), middle (8.10µM), and high (32.4µM) quality control samples.

Sample preparation

Content uniformity determination of the tablet and injection was done as described in the following.

Five ciprofloxacin tablets, two of each brand, were ground into powder and mixed well. The powder, containing approximately 50 mg ciprofloxacin, was measured. It was dissolved and diluted with mobile phase to a ciprofloxacin solution (approximately 8 µg/mL). The ciprofloxacin solution (150 µL) was mixed with 154 µM IS solution 150 µL. The mixture was centrifuged at 10,000 rpm for 5 min. The supernatant (200 µL) was collected and analyzed by HPLC.

The ciprofloxacin injection labeled concentration (2 mg/mL) of three brands was diluted by mobile phase to a ciprofloxacin solution (8 µg/mL). The previously mentioned ciprofloxacin solution (150 µL) was also mixed well with 154 µM IS solution 150 µL. The mixture solution (200 µL) was collected and analyzed by HPLC.

Plasma was prepared from the blood of a laboratory volunteer and stored at –20°C for biological analysis. Plasma samples containing known or unknown concentrations of ciprofloxacin were allowed to thaw at ambient temperature.

Using ACN to precipitate the protein in plasma samples is a simpler clean-up method (10). Each of the 250 µL plasma samples was added to 100 µL of umbelliferone solution as an internal standard, and 150 µL solution of ACN was also added to the plasma sample. The final mixture, which with 50% total ACN content was high enough to allow protein to be precipitated, was vortexed and centrifuged for 15 min at 12,000 rpm. The supernatant (200 µL) was transferred to a vial for HPLC analysis, and an autosampler was used to inject 10 µL of each sample into the HPLC.

For the pharmacokinetic study, approximately 7~8 mL blood were collected from a volunteer with an intravenous catheter, using a blood collection tube at the scheduled times. The blood was centrifuged to plasma after collection. The plasma was conserved at –20°C. The next day, all collected plasma was prepared

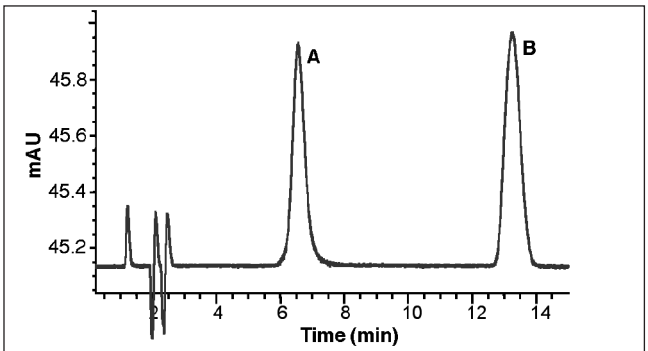

Figure 2. Chromatogram of ciprofloxacin at 8.10 µM in mobile phase. Conditions: Alltima C18 column (4.6 × 150 mm; 5 µm) with Nova-Pak C18 (4 µm) guard column; 2% acetic acid aqueous solution–ACN (84:16, v/v); flow rate: 1.0 mL/min; injection volume, 10 µL. Peaks: ciprofloxacin, A; umbelliferone, B.

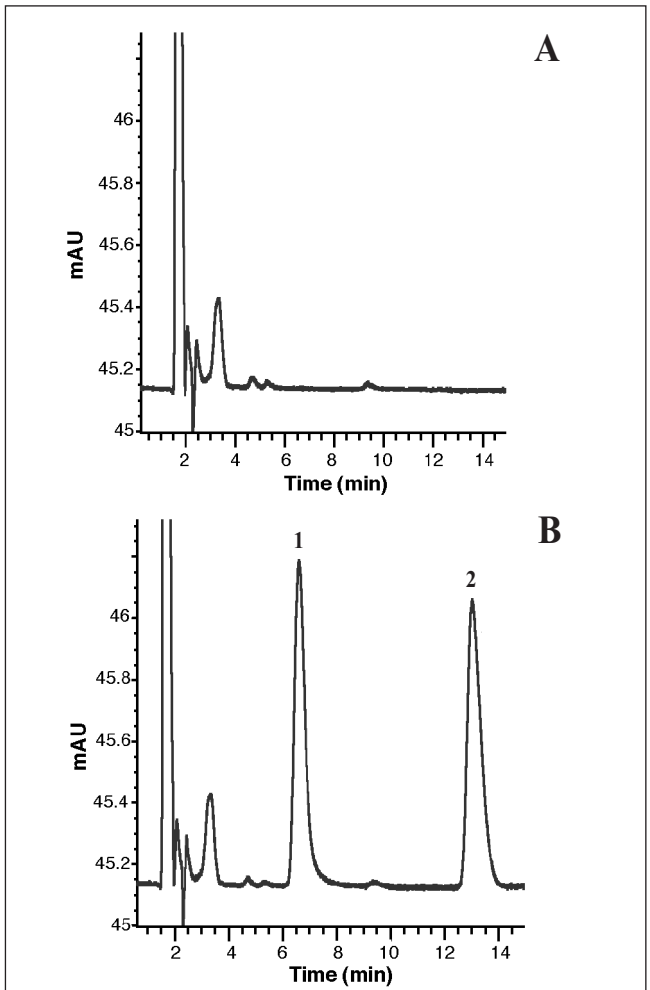

Figure 3. The chromatograms of blank plasma and plasma spiked with ciprofloxacin (8.10 µM) and umbelliferone (77.1 µM). Peaks: ciprofloxacin, 1; umbelliferone, 2.

to the supernatant as in the previously described procedure and injected for HPLC analysis.

Recovery

The recovery for ciprofloxacin in pharmaceutical analysis was calculated at four concentrations (12.96µM, 25.92µM, 38.88µM, and 51.84µM). The absolute recovery of ciprofloxacin was determined at four concentrations (1.01µM, 4.05µM, 16.20µM, and 64.80µM) by the peak area ratio from the supernatant of the plasma sample to a standard ciprofloxacin solution with umbelliferone. The relative recovery was calculated at three different concentrations (2.03µM, 8.10µM, and 32.4µM) based on a daily standard curve.

Stability

The stability of ciprofloxacin was first measured at ambient temperature for 48 h using both standard and plasma samples. The stability of ciprofloxacin during the freeze-thaw procedure needed for sample analyses was further assessed at two different sample concentrations, 8.10 µM and 32.4 µM. The samples were removed from the freezer and allowed to thaw at ambient temperature, then frozen again overnight. This process was repeated three times before the final stability determination was performed in this study.

Table I. Precision and Accuracy for the Determination of Ciprofloxacin in Pharmaceuticals

Concentration known (µM)	Concentration found (µM)*	R.S.D. (%)	R.E. (%)
Intra-day†			
4.05	4.00 ± 0.04	1.02	–1.23
16.20	16.13 ± 0.05	0.30	–0.43
64.80	64.52 ± 0.26	0.40	–0.43
Inter-day‡			
4.05	4.02 ± 0.06	1.52	–3.00
16.20	16.09 ± 0.23	1.44	–0.68
64.80	64.10 ± 0.55	0.86	–1.08

* Mean ± S.D. of five replicate analyses.
† Intraday data was based on five replicate analyses.
‡ Interday data was based on five replicate analyses on five different days.

Table II. Precision and Accuracy for the Determination of Ciprofloxacin Spiked in Human Plasma

Concentration known (µM)	Concentration found (µM)*	R.S.D. (%)	R.E. (%)
Intra-day†			
2.03	2.07 ± 0.06	3.03	1.93
8.10	7.79 ± 0.08	1.08	–3.82
32.40	32.14 ± 0.65	2.02	–0.80
Inter-day‡			
2.03	2.04 ± 0.06	3.35	0.37
8.10	7.63 ± 0.25	3.39	–5.71
32.40	31.95 ± 1.05	3.30	–1.37

* Mean ± S.D. of six replicate analyses.
† Intraday data was based on six replicate analyses.
‡ Interday data was based on six replicate analyses on six different days.

Application

A healthy male volunteer was given a 500 mg ciprofloxacin tablet orally. Blood samples (10 mL each) were obtained at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, and 24 h after oral administration. The concentrations of ciprofloxacin were determined based on the daily standard curve.

Results and Discussion

Chromatography

Figure 2 shows a typical chromatogram of ciprofloxacin standard solution with umbelliferone as an internal standard. There were three component mobile phases required in previous studies (10,12,20,25). Alltima C_{18} phases are polymerically bonded and double endcapped to provide long column lifetimes, even when using harsh mobile phases. This method used a two-component mobile phase because an Alltima C_{18} column was used. The chromatograms of blank plasma and plasma spiked with ciprofloxacin had interferences from the plasma matrix in 2 to 4 min intervals, and were free from any interferences by endogenous substances, especially around the retention times of ciprofloxacin (6.5 min) and umbelliferone (13.2 min) (Figure 3). The chromatographic analysis time of approximately 14 min in this method is somewhat longer than 9 min in a previous work (10). The pretreatment of plasma samples in this method is simpler than the extraction after protein precipitation in the previous work. Their extraction procedure is more complicated and tedious than ours.

Linearity and limit of quantitation

The regression equations of ciprofloxacin in pharmaceutical and biological analysis showed a linear response over the range of 0.51~130µM (n = 5) and 0.51~64.8 µM (n = 6), respectively. The correlation coefficients for the calibration curves were all approaching 0.9999 in inter- and intra-day analysis. The limit of detection (S/N = 3, injection 10 µL) was found to be 0.25µM. Detection of ciprofloxacin in biological samples by HPLC can be managed by UV or fluorescence detection. The limits of quantitation of ciprofloxacin in the literature are as following: 3 nM by fluorescence detection (10), 0.09 µM by UV detection at 275 nm (12), 0.1µM by UV detection at 280 nm (20), and 0.2 µM by fluorescence detection (25).

Table III. Recoveries of Ciprofloxacin in Tablets (n = 3)

Solutions	Ciprofloxacin conc. spiked (µM)	Concentration found* (%)	Recovery (µM)
A	0	25.06 ± 0.18	–
B	12.96	37.65 ± 0.17	97.12
C	25.92	52.22 ± 0.10	104.78
D	38.88	63.54 ± 0.12	98.97
E	51.84	75.12 ± 0.13	96.58

* Mean ± S.D. of triplicate analyses by standard addition method.

Accuracy and precision

Accuracy and precision were executed with five and six replicates in pharmaceutical and biological analysis (Tables I and II, respectively). For pharmaceutical analysis, accuracy, calculated as RE, was lower than 3.00%. Precision, determined as RSD, was lower than 1.52%. Biological analysis, accuracy calculated as RE, was lower than 5.71%. Precision, determined as RSD, was lower than 3.39%. The values obtained for the accuracy and precision of the previous methods (10,12,20,25) were all lower than 10% and 16.31%, respectively.

Recovery

The recoveries of ciprofloxacin in tablets, as shown in Table III, indicated that the recovery was more than 96.5%. The absolute and relative recoveries for ciprofloxacin spiked in human plasma were all greater than 93.8% and 96.1%, respectively (Tables IV and V). In one study (25), percent recoveries of ciprofloxacin from plasma were within the range of 78~81%. Sample preparation of this method was direct injection of the supernatant from plasma after protein precipitation with ACN and acetic acid. In a recent study (12), the mean recovery value was 93.5% for ciprofloxacin in serum samples. It also used direct injection of reconstituted solution which was made from the residue from the supernatant of human blood serum after protein precipitation with ACN.

Stability

Figures 4 and 5 contain our stability studies of ciprofloxacin at ambient temperature for pharmaceutical and biological samples, respectively. It is clear that no degradation was observed in either or these conditions over a 48 h period. Furthermore, ciprofloxacin is stable even under the freeze-thaw cycles performed in the current study. The results of these studies are summarized in Table VI.

Table IV. Absolute Recovery of Ciprofloxacin in Human Plasma

Concentration spiked (μM)	Absolute recovery (%)*
1.01	95.75 ± 3.56
4.05	95.89 ± 1.37
16.20	94.79 ± 0.08
64.80	93.87 ± 0.35

* Mean ± S.D. of triplicate analyses.

Table V. Relative Recovery of Ciprofloxacin in Human Plasma

Concentration spiked (μM)	Concentration found (μM)*	Recovery (%)
2.03	2.06 ± 0.06	101.93
8.10	7.79 ± 0.08	96.17
32.40	32.14 ± 0.65	99.20

* Mean ± S.D. of six replicate analyses.

Selectivity

A solution containing seven different quinolones (i.e., pipemidic acid, norfloxacin, lomefloxacin, moxifloxacin, ciprofloxacin, levofloxacin, and ofloxacin) was used to validate the selectivity of our analytical method. The result is shown in Figure 6. A peak of three quinolones (norfloxacin, ofloxacin, and levofloxacin) appears to partially overlap with ciprofloxacin in this condition. Because levofloxacin is an enantiomer of ofloxacin, it was not surprising that ofloxacin and levofloxacin were in a peak and not separated. The structure of norfloxacin is close to ofloxacin, and they, too, did not separate in this condition. In a previous study (15), a good selectivity was shown by a good resolution in six quinolones (cinoxacin, levofloxacin, ciprofloxacin, gatifloxacin, moxifloxacin, trovafloxacin). However, the structures of these six quinolones were quite different. It is unlikely that an individual would take more than one quinolone at a time in a pharmacokinetic study. This method is suited to be applied for the determination of ciprofloxacin. However, the good resolutions of each of these quinolones could be further improved by using a smaller ACN proportion of the mobile phase.

Application

The application of this methodology for the content uniformity analyses of five brands of ciprofloxacin from different commercial sources was further assessed. The results of these studies are summarized in Tables VII and VIII for two brands of tablets and three brands of injections. Using the USP's regulation standard, all of these products were found to be within the allowable limit of 90.0% to 110.0%.

The applicability of our method in the determination of ciprofloxacin levels in human plasma for a pharmacokinetic study was also studied. We did not need to change a guard

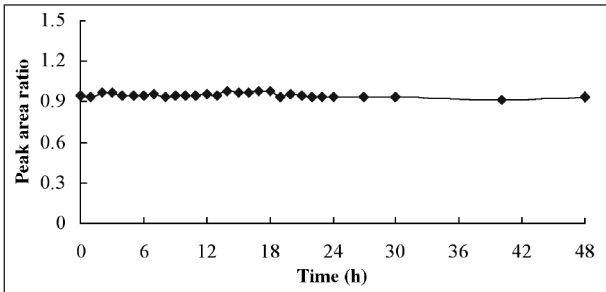

Figure 4. Stability of ciprofloxacin in mobile phase matrix.

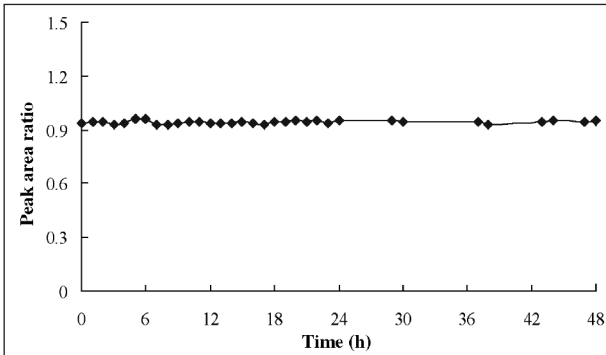

Figure 5. Stability of ciprofloxacin in human plasma.

Table VI. Plasma Freeze-Thaw Cycle Results

Sample (μM)	N	Cycle 1 Measured* (μM)	Cycle 1 R.E. (%)	Cycle 2 Measured* (μM)	Cycle 2 R.E. (%)	Cycle 3 Measured* (μM)	Cycle 3 R.E. (%)
8.10	3	8.50 ± 0.02	4.91	8.46 ± 0.24	4.41	8.44 ± 0.30	4.26
32.4	3	31.35 ± 0.81	–3.22	31.09 ± 0.41	–4.03	31.29 ± 0.36	–3.41

* Mean ± S.D. of triplicate analyses.

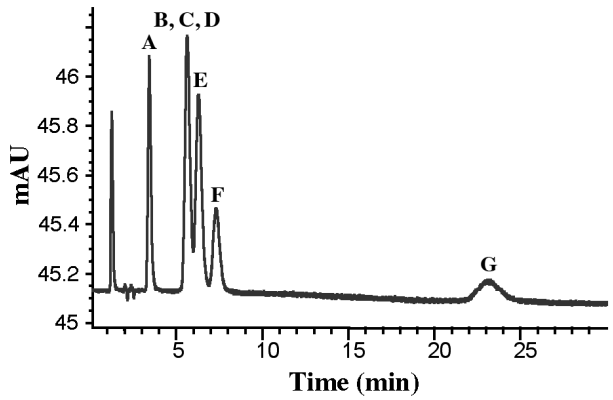

Figure 6. The chromatogram of selectivity study. Peaks: pipemidic acid, A; norfloxacin, B; ofloxacin, C; levofloxacin, D; ciprofloxacin, E; lomefloxacin, F; moxifloxacin, G.

Table VII. Analytical Results for Content Uniformity of Ciprofloxacin Film-Coated Tablets from Commercial Sources

Sample*	Amount found† (mg)	Percentage of claimed content‡ (%)
Brand A	250.46 ± 1.73	100.18
Brand B	266.16 ± 1.76	106.49

* The labeled concentration of ciprofloxacin in each tablet was 250 mg.
† Mean ± S.D. of fifteen replicate analyses.
‡ A content uniformity test was used to check the variation of ciprofloxacin in each tablet.

Table VIII. Analytical Results for Content Uniformity of Ciprofloxacin Injections from Commercial Sources

Sample*	Amount found† (mg)	Percentage of claimed content‡ (%)
Brand C	2.00 ± 0.01	100.07
Brand D	2.01 ± 0.02	100.61
Brand E	2.06 ± 0.02	101.36

* The labeled concentration of ciprofloxacin in each solution was 2 mg/mL.
† Mean ± S.D. of fifteen replicate analyses.
‡ A content uniformity test was used to check the variation of ciprofloxacin in each solution.

column after 360 injections of plasma sample by this simple plasma clean-up procedure. With a healthy male volunteer receiving a single 500 mg ciprofloxacin tablet, the plasma concentration of ciprofloxacin over a 24 h period was monitored. Figures 7A and 7B show 0.5 and 12 h, respectively, chromatographic analyses of ciprofloxacin. From our study, at time 0 h there was an intercept of concentration from linear regression equation; it was clear that the time required for ciprofloxacin to reach maximum concentration was 0.5 h, and the maximum plasma level was determined to be 4.46 μM as shown in Figure 8. The previously published observations were shown as 0.9 h, 3.3μM (18) and 1.2~2.0 h, 4.6~5.2μM (20).

Conclusion

A simple HPLC method with UV detection for the analysis of ciprofloxacin was developed in our laboratories. The method is simple because the mobile phase was without buffer solutions or ion-pairing reagents and used only two solvents, aqueous acetic acid and ACN. It provided linearity, precision, and accuracy in its measurement. It also requires a very simple sample preparation

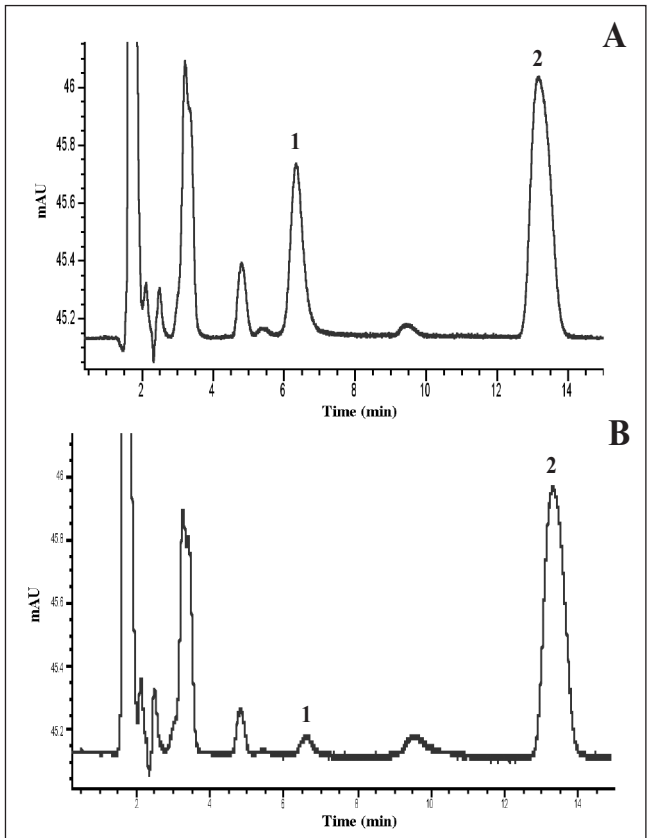

Figure 7. Chromatogram of a healthy volunteer 0.5 h after receiving a 500 mg ciprofloxacin tablet. Peaks: ciprofloxacin, 1; umbelliferone, 2 (A). Chromatogram of a healthy volunteer 12 h after receiving a 500 mg ciprofloxacin tablet. Peaks: ciprofloxacin, 1; umbelliferone, 2 (B).

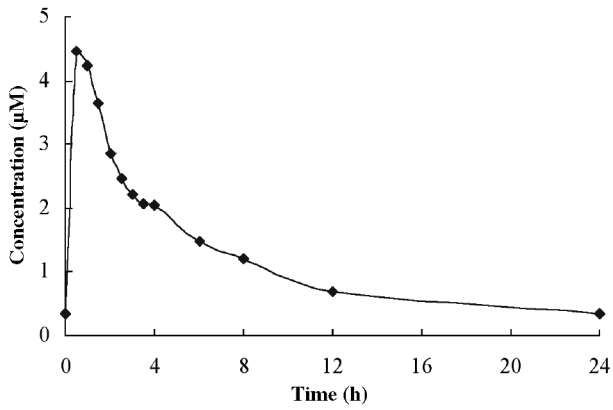

Figure 8. Concentrations of ciprofloxacin at different sampling times after a single oral dose to a healthy volunteer (male, 24 years, 65 kg).

without the need for pretreatment, which includes an extra dichloromethane or solid-phase extraction step in sample preparation. Although the internal standard, umbelliferone, was chosen considering its retention time, the method could possibly use fluorescence detection.

This method has been successfully applied to the determination of ciprofloxacin in pharmaceuticals and plasma of a volunteer after oral administration of a 500 mg ciprofloxacin tablet in a pharmacokinetic study. The assay will be applied to the pharmacokinetic study of patients with ciprofloxacin treatment.

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Manuscript received December 5, 2006;
revision received October 29, 2007.