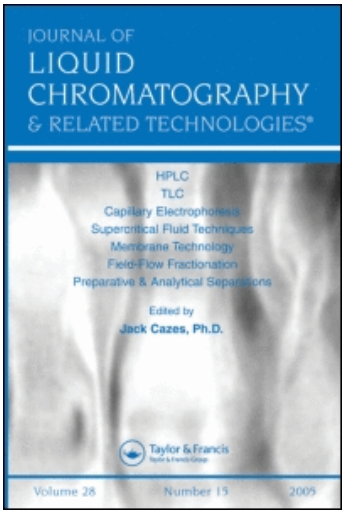

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VALIDATED DETERMINATION OF CIPROFLOXACIN IN INFLUENZA VACCINE BY RP-HPLC

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic method for the determination of ciprofloxacin in influenza vaccine has been developed. The stationary phase was a Purospher RP-18e column (125 × 3.5 mm; 5 μm), the mobile phase consisted of acetonitrile/water/phosphoric acid (85%) (15 : 85 : 0.25, v/v/v %), and its pH was adjusted to pH 3.00 using distilled triethylamine immediately before use. Separation was achieved using a flow rate of 0.6 mL/min at ambient temperature. The ciprofloxacin was detected at 280 nm. The retention time for ciprofloxacin was 4.60 ± 0.15 min. The limit of detection was 2 ng/mL; the limit of quantification was found to be 5 ng/mL.

INTRODUCTION

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolone carboxylic acid], shown in Figure 1, is one of the fluoro-

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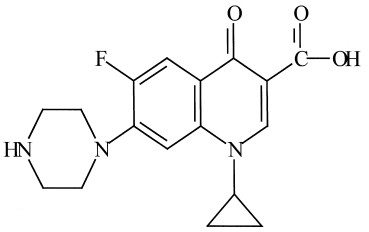

Figure 1. Structure of ciprofloxacin.

quinolones, a group of antibiotics that have been shown to have an extended antibacterial spectrum. Ciprofloxacin is effective against a wide range of infections, e.g., of the urinary tract, respiratory tract, and gastrointestinal tract (1,2).

A number of liquid chromatographic methods have been developed for the determination of ciprofloxacin and its metabolites in human serum, urine, saliva, and sputum (1,3,4), in ocular aqueous humor (5), in human hair (6), and in rat plasma (7), and bovine milk and plasma (8). These methods are suitable for the measurement of the drug level in human or animal specimens after oral or intravenous intake.

This paper describes a novel method that allows determination of ciprofloxacin as an active ingredient in influenza vaccine or in other pharmaceutical products.

EXPERIMENTAL

Chemicals

Acetonitrile, gradient grade, and orthophosphoric acid (85%), analytical grade, were obtained from Merck (Darmstadt, Germany). Water was obtained from a Millipore Milli-Q system (Waters, Milford, MA, USA). Triethylamine (Riedel-de Haën, Seelze, Germany) was freshly distilled before use. Ciprobay infusion for intravenous use (S) (containing 2 mg/mL of ciprofloxacin) was obtained from Bayer AG (Leverkusen, Germany).

The matrix solution (M) consisted of the following: 10.40 g of Na_3PO_4, 3.07 g of NaCl, 0.07 g of KCl, 0.57 g of KH_2PO_4, 0.35 g of Na_2HPO_4, 0.09 g of merthiolate, and 5.40 g of $AlCl_3$ diluted to 1000 mL with distillated water (Millipore).

Apparatus

The separation was performed using isocratic elution at a flow rate of 0.6 mL/min by a series 200 LC pump (Perkin-Elmer, Norwalk, CT, USA) attached to an ISS 200 autosampler (Perkin-Elmer). The stationary phase was a Purospher RP-18e column (125 × 3.5 mm; 5 μm; Merck). The ultraviolet detection was carried out at 280 nm using a 235°C diode array detector (Perkin-Elmer).

The software was Turbochrom Navigator from Perkin-Elmer. The mobile phase was ultrasonicated in a Realsonic 57 ultrasonic bath, and its pH was adjusted using a Jenway 3020 pH meter.

Sample Preparation

Sample preparation consisted of mixing equal volumes of influenza vaccine solution (Fluval) with the mobile phase followed by filtering through a 0.45-μm filter (Millipore). The filtrate was then injected onto the chromatographic system. The stationary phase was a Purospher RP-18e column (125 × 3.5 mm; 5 μm); the mobile phase consisted of acetonitrile/water/phosporic acid (85%) (15:85:0.25, v/v/v %). Because the retention time for ciprofloxacin was found to be considerably affected by the acetonitrile content of the mobile phase, the components were measured by mass. The pH was adjusted to pH 3.00 using distilled triethylamine immediately before use. The acetonitrile content of the mobile phase is relatively low (15 v/v %); hence, the vaporization of the mobile phase causes significant error. Therefore, remodification of the mobile phase is required from time to time to avoid the shift in retention time. Separation was achieved using a flow rate of 0.6 mL/min at room temperature. Ultraviolet detection was carried out at 280 nm.

Validation

Linearity

Stock solutions (T1 and T2) were prepared from the Ciprobay solution (S) (containing 2 mg/mL ciprofloxacin) by dilution with the eluent as follows. For T1 solution, 25 μL of S solution was diluted to 250 mL with the eluent (0.2 μg/mL ciprofloxacin). For T2 solution, 100 μL of S solution was diluted to 100 mL with the eluent (2 μg/mL ciprofloxacin).

Standard solutions (2–500 ng/mL) were prepared from stock solutions (T1 and T2), the eluent, and the matrix solution (M) as shown in Table 1. Every stan-

Table 1. Calibration Standards for Ciprofloxacin

T1 Solution (µl)	T2 Solution (µl)	Mobile Phase (µl)	M Solution (µl)	Concentration (ng/ml)
50	—	950	1000	5
100	—	900	1000	10
250	—	750	1000	25
500	—	500	1000	50
—	100	900	1000	100
—	200	800	1000	200
—	500	500	1000	500

dard solution was filtered through a 0.45-µm Millipore membrane and then was injected onto the chromatographic system.

Specificity

First, 1000 µL of M solution and 1000 µL of the eluent were mixed, filtered through a 0.45-µm Millipore membrane, and then injected onto the chromatographic system (background).

Next, 1000 µL of M solution, 250 µL of T1 solution, and 750 µL of the eluent were mixed, filtered through a 0.45-µm Millipore membrane, and then injected onto the chromatographic system (25 ng/mL ciprofloxacin sample). A clear analytical window was expected on the background chromatogram at the retention time of ciprofloxacin. The dead time was determined using a mixture of 1000 µL of distilled water and 1000 µL of eluent. The first significant peak was accepted as dead time. The net retention time was calculated as the difference between retention time and dead time. For the characterization of robustness, the net retention time was used.

Repeatability

To determine repeatability, 1000 µL of M solution, 100 µL of T1 solution, and 900 µL of the eluent were mixed, filtered through a 0.45-µm Millipore membrane, and then injected onto the chromatographic system five times, consecutively (10 ng/mL ciprofloxacin sample). Mean retention time and peak area, as well as the precision for retention times and peak areas, were calculated. The

method is repeatable if the relative standard deviation for retention times is lower than 1% and for peak areas is lower than 10%.

Accuracy

First, 1000 μL of M solution, 100 μL of T1 solution, and 900 μL of the eluent were mixed, filtered through a 0.45-μm Millipore membrane, and then injected onto the chromatographic system five times, consecutively [10 ng/mL ciprofloxacin matrix sample (no. 1)].

Next, 40 μL of T1 solution and 760 μL of the eluent were mixed and then injected onto the chromatographic system five times, consecutively [10 ng/mL ciprofloxacin sample (no. 2)]. Recovery was calculated as the quotient of peak areas: no. 1/no. 2. The method is reproducible if recovery (R%) is 100 ± 5%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

For these, 1000 μL of M solution and 50, 40, 30, 20, or 10 μL of T1 solution were mixed, diluted to 2000 μL with the eluent (5, 4, 3, 2, and 1 ng/mL), filtered through a 0.45-μm Millipore membrane, and then injected onto the chromatographic system.

LOD was determined as the concentration at which the peak height had a signal/noise ratio of 3; LOQ was determined as the concentration at which the signal/noise ratio was 10.

Robustness

The effect of acetonitrile content and pH of the mobile phase for the retention time/retention factor was investigated.

The acetonitrile content was set at 12–18 v/v % and net retention time for ciprofloxacin was determinated.

The pH of the mobile phase was adjusted to pH 2.3 or 4, consecutively, using distillated triethylamine. Then retention times were measured, and retention factors were calculated.

RESULTS

Under the chromatographic conditions described above, the retention time for ciprofloxacin was 4.55 ± 0.15 min. (Fig. 2). There was no interfering peak

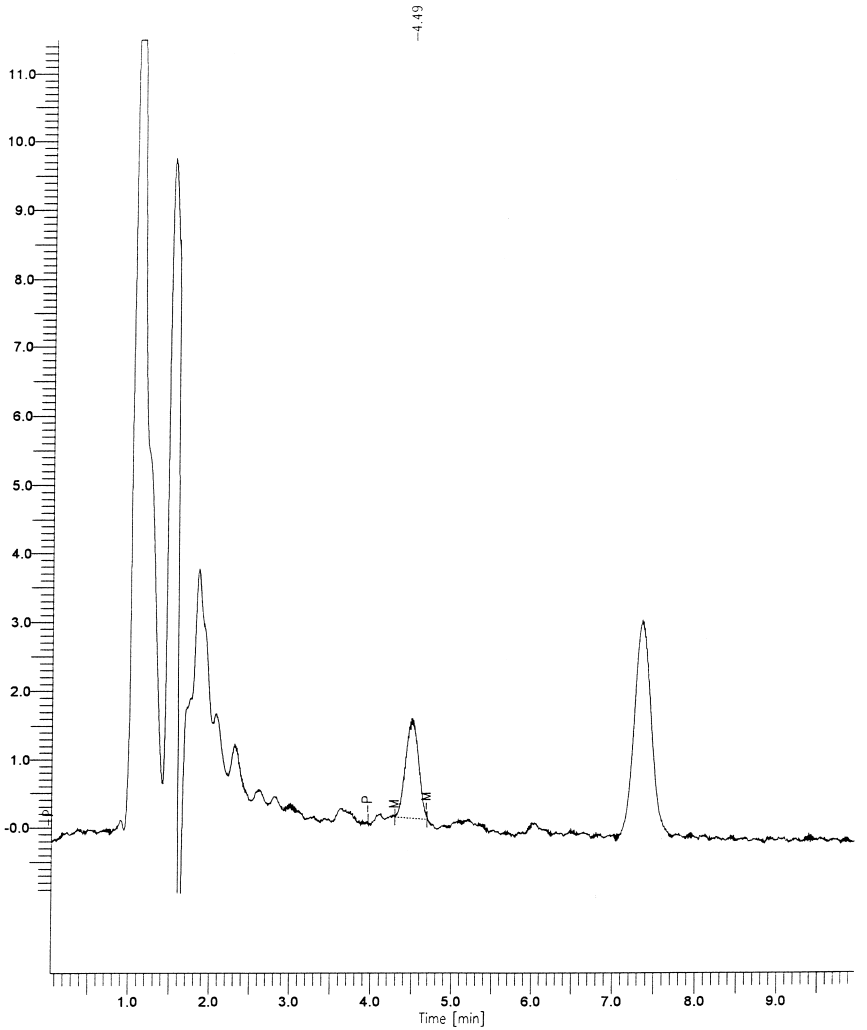

Figure 2. Chromatogram for the 25 ng/mL ciprofloxacin sample.

found on the background chromatogram (Fig. 3) at the retention time of ciprofloxacin; the determination is specific. The calibration curve (Fig. 4) showed good linearity in the concentration range of 2–500 ng/mL ($A = 288.59 \cdot c + 3614.9$; $R^2 = 0.9950$).

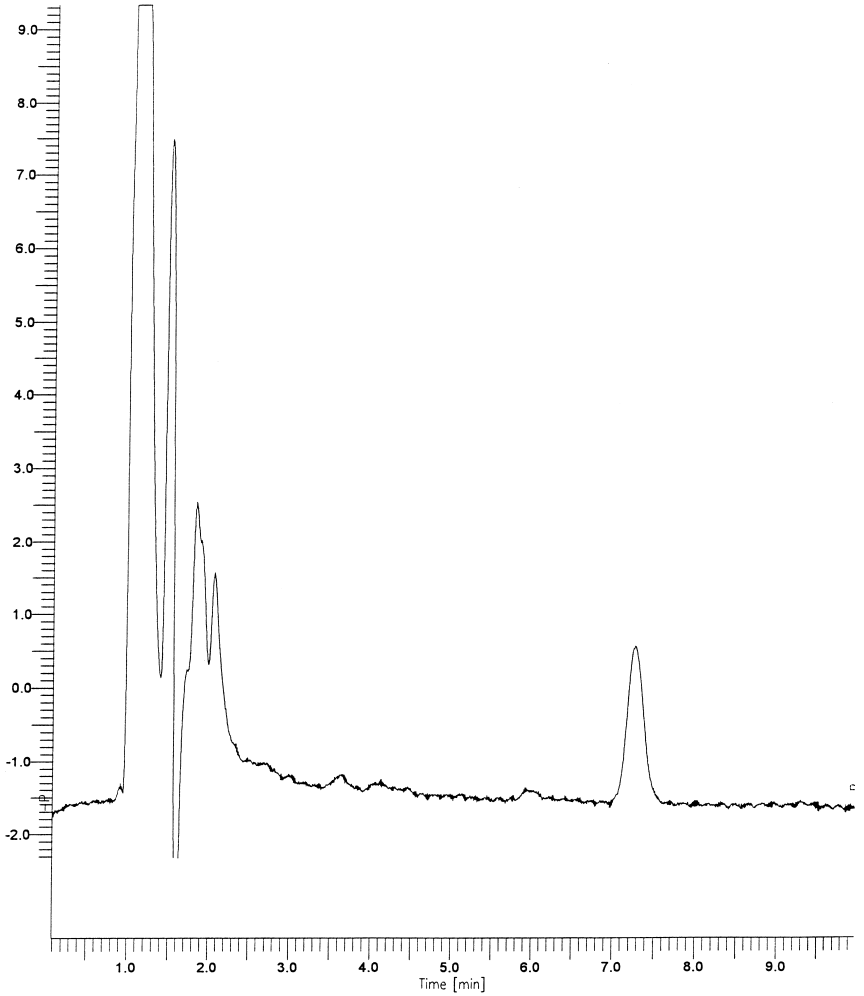

Figure 3. Background chromatogram for ciprofloxacin.

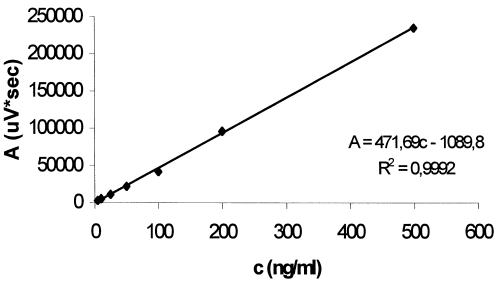

Figure 4. Calibration curve for ciprofloxacin.

For the 10 ng/mL ciprofloxacin sample, the mean peak area calculated from five parallel measurements was 5111 with a relative standard deviation of 9.2%; the retention time calculated from five measurements was 4.55 with a relative standard deviation of 0.5%; hence, the method is repeatable. Because the recovery for the 10 ng/mL ciprofloxacin samples calculated from five measurements was found to be 95.7%, the method is accurate.

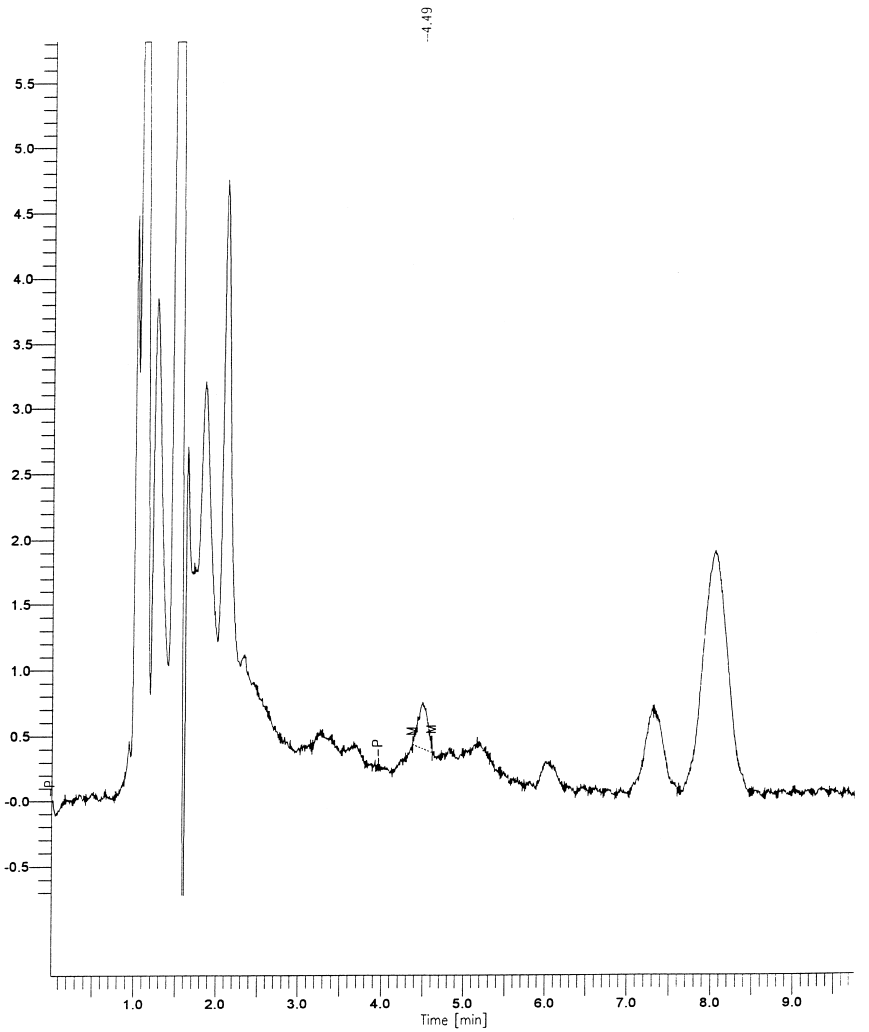

Figure 5. Chromatogram for the 5 ng/mL ciprofloxacin sample (LOQ).

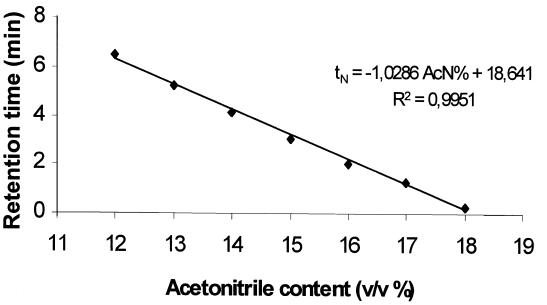

Figure 6. The effect of acetonitrile (ACN) content of the mobile phase for the retention time.

The LOD at a signal/noise ratio of 3 was 2 ng/mL. The LOQ at a signal/noise ratio of 10 was found to be 5 ng/mL (Fig. 5).

The retention time for ciprofloxacin was found to be considerably affected by acetonitrile content of mobile phase (Fig. 6); therefore, components must be measured by mass, and loss of acetonitrile by vaporization must be replaced. Though the effect of pH on the retention factor is not significant (Fig. 7), the pH of the eluent must be adjusted exactly to pH 3.00 using distilled triethylamine immediately before use.

DISCUSSION

A specific, repeatable, and accurate method for the detection of ciprofloxacin has been developed. This highly sensitive procedure is suitable for the rapid and simple determination of low concentations of ciprofloxacin in

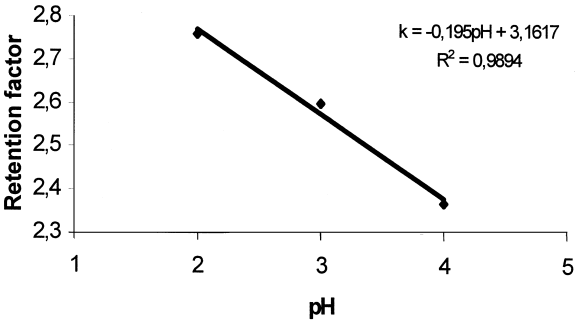

Figure 7. The effect of pH of the mobile phase for the retention factor.

influenza vaccine or in other pharmaceutical products. Accurate mixing of mobile phase components by mass and replacement of evaporated acetonitrile is essential to guarantee robustness.

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