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Short communication

Determination of grepafloxacin in plasma and urine by a simple and rapid high-performance liquid chromatographic method

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Abstract

A rapid, specific, sensitive and economical method has been developed and validated for the determination of grepafloxacin in human plasma and urine. The assay consisted of reversed-phase HPLC with UV detection. Plasma proteins were removed by a fast and efficient procedure that has eliminated the need for costly extraction and evaporation. For the urine samples, the only required sample preparation was dilution. Separation was achieved on a reversed-phase TSK gel column with an isocratic mobile system. The method had a quantification limit of 0.05 μg/ml in plasma and 0.5 μg/ml in urine. The coefficients of variation (C.V.) were less than 4% for within- and between-day analyses. The method was successfully applied to a pharmacokinetic study, and was proved to be simple, fast and reproducible. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Grepafloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-5-methyl-7-[3-methyl-1-piperazinyl]-4-oxo-3-quinoline carboxylic acid monohydrochloride sesquihydrate) [OPC-17116] (Fig. 1) is a new fluoroquinolone that is currently being developed for the treatment of lower respiratory tract infections and sexually transmitted diseases [1–3]. Few high-performance liquid chromatographic methods in the literature are reported to allow the quantification of grepafloxacin [4–6]. The HPLC assays published by Efthymiopoulos et al. [4] and Mukai et al. [5] share the disadvantage of utilizing large plasma sample volume, complex extraction procedures and expensive fluorescence detection. Recently we have reported a HPLC method with UV detection for the simultaneous determination of grepafloxacin, ciprofloxacin, and theophylline in human plasma and urine [6]. This assay allowing these drugs to elute and be resolved in a single chromatogram, facilitates detailed investigations on the interaction between these two quinolones and theophylline. The reported method also can be used for the determination of only grepafloxacin, but the extraction procedure involved and relative long retention time for grepafloxacin make it of limited use.

Since further worldwide, well-planed clinical studies are now needed to confirm the position of

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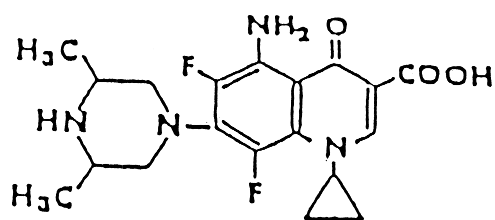

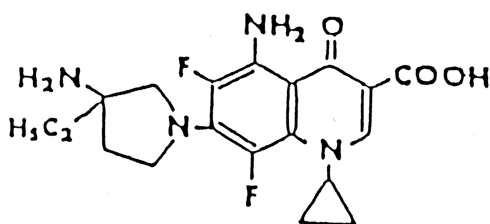

Fig. 1. Chemical structures of grepafloxacin and internal standard AT-4117.

grepafloxacin in relation to other fluoroquinolones, any methodological improvements that can result in reduced costs and preparation times for samples are attractive.

The objective of this study was to develop a simple, rapid, sensitive and economical method for the determination of grepafloxacin in plasma and urine.

2. Experimental

2.1. Chemicals and materials

Chemicals, methanol, acetonitrile, acetic acid and *N*,*N*-dimethylformamide, were obtained from Wako Pure Chemical Industries (Osaka, Japan). Grepafloxacin was kindly donated by Otsuka Pharmaceuticals (Tokyo, Japan), and AT-4117 (5-amino-7-[3-amino-3-ethyl-1-pyrrolidinyl]-1-cyclopropyl-6,8-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid) (Fig. 1) used as an internal standard (I.S.) was obtained from Shionogi Pharmaceutical Co. (Osaka, Japan). All reagents used were of analytical or HPLC grade.

2.2. Chromatographic system and conditions

A Shimadzu model LC-6A liquid chromatographic system equipped with a SIL-6A auto sampler (Shimadzu, Analytical Instruments Division, Tokyo, Japan) and a UV-8010 spectrophotometer (TOSOH, Scientific Instrument Division, Japan) were used for analyses. The output of the detector was monitored by a chromatocorder 12 (SIC System Instrument Co., Ltd, Japan). Separation was accomplished using a reversed-phase TSK gel column (octadecylsilane-80TM; particle size, 5 μm; 150×4.6 mm I.D. (TOSOH Corporation, Japan). The column was protected with a pre-column (Guard-Pak) filled with a μBondapac C_{18} cartridge (Merck kGaA, Darmstadt, Germany).

The chromatographic separation was performed at 35°C, using an isocratic mode. The mobile phase composition consisted of a mixture of acetic acid 5%–acetonitrile–methanol (70:15:15 v/v). To improve chromatographic characteristics 1% (v/v) triethylamine was added to the mobile phase. The flow-rate was adjusted at 0.8 ml/min. The UV detector was set at 296 and 340 nm for plasma and urine, respectively, and the sensitivity was set at 0.05 absorbance units full scale. The chart speed was 2 cm/min.

2.3. Stock solutions and standards

Stock solutions of grepafloxacin and I.S. (1 mg/ml each) were prepared separately in methanol–water (1:1 v/v). These stock solutions were diluted with methanol–water, drug-free plasma, or urine (1:1 v/v) to yield aqueous, plasma, and urine working standards, respectively. Standards for the calibration curves were prepared from 0.05 to 2.5 μg/ml in drug-free control plasma and from 0.5 to 500 μg/ml in drug-free control urine.

2.4. Sample preparation

2.4.1. Plasma

To 0.5 ml of plasma sample or standard, 50 μl of the I.S. working solutions (5 μg/ml) was added. The sample was vortex-mixed for 30 s and 0.5 ml of *N,N*-dimethylformamide were added, and the mixture was again vortex-mixed for 30 s and incubated at 50°C for 10 min. Then, the mixture was centrifuged at 8000 *g* for 2 min at ambient temperature in a microcentrifuge. The supernatant was diluted with an equal volume of water and 50 μl of the sample was injected into the HPLC.

2.4.2. Urine

The urine samples were diluted 1:4 (v/v) with distilled water. In a microcentrifuge tube, 50 μl of the working solution of I.S. was added to 100 μl of the diluted urine. The mixture was vortex-mixed, and 20 μl was injected directly into the HPLC system.

3. Results and discussion

Typical chromatograms obtained by the described method are shown in (Fig. 2). Grepafloxacin and I.S. peaks were eluted with retention times of ~11 and 15 min, respectively. The base line was relatively free from drift. Endogenous interference from plasma was not detected. The standard curves, with grepafloxacin concentrations ranged from 0.05 to 2.5 μg/ml in plasma and from 0.5 to 500 μg/ml in urine. To determine linearity, six standards were prepared by adding known amounts of grepafloxacin

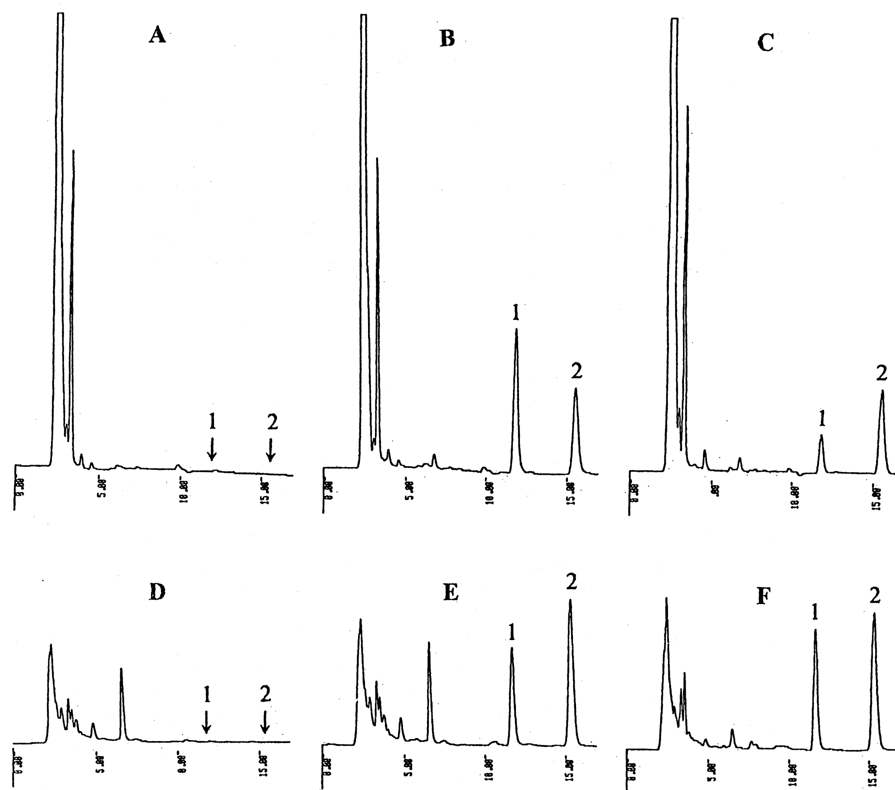

Fig. 2. Representative chromatograms of (A) plasma blank, (B) plasma standard (1 μg/ml), (C) plasma sample collected 12 h after a 300-mg oral dose of grepafloxacin, (D) urine blank, (E) urine standard (5 μg/ml), and (F) urine sample collected 24 h after a 300-mg oral dose of grepafloxacin. Peaks: 1, grepafloxacin; 2, internal standard (AT-41417).

to the drug-free plasma or urine. The calibration curves were obtained by least-squares linear fitting of the drug-to-internal standard ratios of peak areas versus the respective drug concentrations. Basic statistics of simple linear regression and correlation were performed. The sum of squares for residuals was equal to zero which indicates that the calibration line was a perfect fit to the data. The statistical parameters, a and b, the y-intercept, and the slope of the regression line, respectively, were calculated by use of appropriate equations [7]. The following equations were derived from the calibration curves: $y = 0.6952x + 0.0016$ $(r = 0.9999)$ and $y = 0.1242x - 0.0126$ $(r = 0.9999)$ for grepafloxacin in plasma and urine, respectively (where y is the peak-area ratio (in arbitrary units) and x is the concentration (in μg/ml)). The standard error of the estimate Sy/x was 0.0065 for grepafloxacin in plasma and 0.142 for grepafloxacin in urine. Variability of the estimated slopes and intercepts was determined by standard deviations of estimated slopes and intercepts and the confidence intervals for the true slopes and intercepts. The standard deviation of the estimated slope, s_b, and intercept, s_a, of the linear-regression lines were 0.005 and 0.007 for grepafloxacin in plasma and 0.032 and 0.079 for grepafloxacin in urine, respectively. The two-tailed value for student's t-test where 95% for the confidence interval was chosen, was used to calculate the confidence limits for the slope β, (0.6952 ± 0.0142) and (0.1249 ± 0.0089) and the intercepts α, (0.0016 ± 0.0019) and (-0.0126 ± 0.219) for grepafloxacin in plasma and urine, respectively.

A standard curve was generated for each analytical run and was used to calculate the concentrations of the drug in unknown samples assayed with that analytical run. The standard curves covered the entire range of expected concentrations. The limit of detection, based on a signal-to-noise ratio of 3:1, was 0.025 and 0.2 μg/ml for grepafloxacin in plasma and urine, respectively. The limit of quantification as determined by precision and accuracy and where the coefficients of variation of $<10\%$ were targeted was 0.05 μg/ml for plasma and 0.5 μg/ml for urine. The specificity of the assay was established with six independent sources of the same matrix. The within-day and between-day coefficients of variation values are summarized in Tables 1 and 2. The mean recoveries of grepafloxacin and the I.S. determined at concentrations ranging from 0.05 to 500 μg/ml were similar and consistent and ranged from 95.79 to 98.64 for grepafloxacin and from 96.84 to 97.56 for the I.S..

In addition, the following drugs at concentrations that exceed or meet levels typically found in patients' plasma specimens were carried through the assay and were found to not interfere: the antiarrhythmics lidocaine, digoxin, disopyramide; the anti-

Table 1
Within-day precision of grepafloxacin determination in plasma and urine[a]

Theoretical concentration μg/ml	Found concentration (SD) μg/ml	C.V., %
Plasma		
0.05	0.051(0.020)	3.79
0.5	0.478(0.090)	1.89
2.5	2.514(0.030)	1.20
Urine		
0.5	0.546(0.013)	3.02
100.0	101.666(0.604)	0.59
500.0	504.043(2.434)	0.48

[a] Analysis was done five times in plasma and urine. SD, standard deviation; C.V., coefficient of variation.

Table 2
Between-day precision of grepafloxacin determination in plasma and urine[a]

Theoretical concentration μg/ml	Found concentration (SD) μg/ml	C.V., %
Plasma		
0.05	0.052(0.002)	3.87
0.1	0.099(0.002)	2.13
0.5	0.486(0.016)	3.21
1.0	0.976(0.017)	1.76
2.0	2.034(0.032)	1.58
2.5	2.505(0.029)	1.17
Urine		
0.5	0.519(0.011)	2.15
5.0	4.925(0.074)	1.50
10.0	9.662(0.109)	1.13
50.0	49.802(1.024)	2.06
100.0	101.381(1.034)	1.02
500.0	499.270(0.960)	0.19

[a] Analysis was done five times in plasma and urine. SD, standard deviation; C.V., coefficient of variation.

biotics amikacin, gentamicine, ciprofloxacin, lovefloxacin, sparfloxacin, ofloxacin; antidepressants amitriptyline, doxepin; antiepileptics carbamazepine, phenobarbital; and the other drugs acetaminophen, caffeine, cyclosporine, prednisolone, theophylline and methotrexate.

The method described is simple, easy to perform, and has the requisite sensitivity for the detection and quantification of grepafloxacin in clinical samples. Compared with the previous method reported [4–6] this assay offers several advantages. One-step protein precipitation is one of the unique features of the procedure described. Although the extraction procedure previously reported [4–6] might reduce the interference from the sample matrices, the cumbersome sample preparation involved limits the clinical usefulness of such a procedure. A number of methods can be used to precipitate proteins from biologic matrices such as using organic solvents, heat treatment, or acidification [8]. Precipitation of proteins by the addition of *N*,*N*-dimethylformamide produced a good recovery and was not associated with column clogging.

The method also takes advantage of grepafloxacin absorption characteristics by using an appropriate detection wave length of 296 nm; this eliminated most of the interference from plasma. Based on expected concentrations, the knowledge that the degree of sensitivity needed for plasma was not necessary for urine, and the need to reduce interference of endogenous materials, the detector was set at 340 nm during the measurement of grepafloxacin concentrations in the urine samples. The requisite sensitivity obtained with a UV detector, allowed us to avoid using a fluorescence detector which is more expensive.

Maintaining the analytical column at 35°C insures reproducible separation and requires lower concentrations of organic solvents. The assay had a stable base line, allowing the automatic injection and running of a large number of samples. The mobile phase used in our method is not expensive and of very simple composition, which gives a longer lifetime of the column and lowers the risk of protein precipitation associated with the use of sodium sulfate in the solvent system. The present method has been used extensively for measuring grepafloxacin in the plasma and urine of healthy volunteers from a pharmacokinetic study. The concentration-time profile for grepafloxacin in plasma after administration of a single 300 mg oral dose to a healthy volunteer is shown in Fig. 3.

In conclusion, the HPLC method described is reproducible, selective, sensitive, and accurate. The

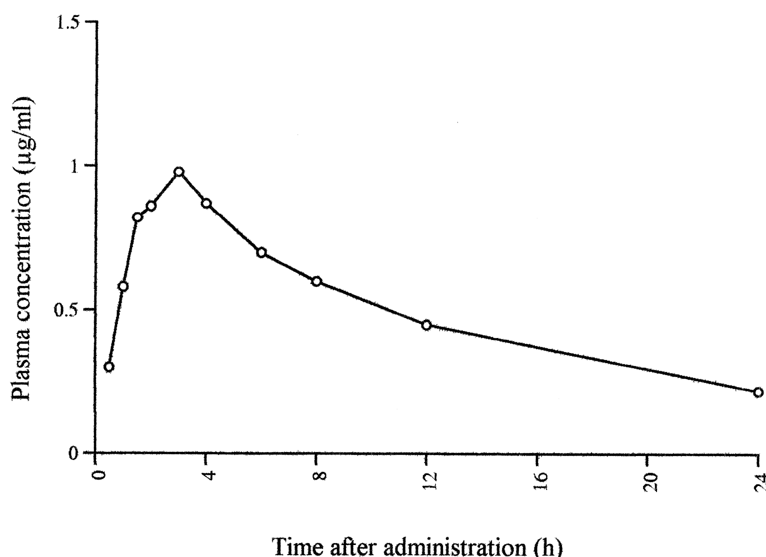

Fig. 3. Concentration-time profile for grepafloxacin in plasma after administration of a single 300 mg oral dose to a healthy volunteer. (The plasma concentrations of the drug were measured using the method described in Section 2).

method is also economical, rapid and very simple. It is suitable for pharmacokinetic studies of grepafloxacin.

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