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Short communication

Determination of gatifloxacin in human serum and urine by high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple and sensitive HPLC method for the determination of gatifloxacin concentrations in human serum and urine was developed and validated. Serum proteins were removed by ultrafiltration through a filtering device after adding a displacing agent. Urine samples were diluted with mobile phase prior to injection. Separation was achieved with a C18 reverse-phase column and gatifloxacin concentrations were determined using ultraviolet detection. The quantitation limits of the assay were 100 ng/ml in serum and 1.0 μg/ml in urine. The assay method was successfully applied to a pharmacokinetic study of gatifloxacin in healthy volunteers.

Keywords: Gatifloxacin

1. Introduction

Since their introduction into clinical practice, fluoroquinolone antibiotics have become widely accepted for the treatment of many bacterial infections in hospitalized patients, out-patients, and nursing home patients. Advanced-generation fluoroquinolones, such as gatifloxacin, offer several advantages over previous agents, including enhanced in vitro activity against clinically important pathogens and improved pharmacokinetics. These advantages result in enhanced pharmacodynamics, which may improve patient outcomes against certain bacterial pathogens, especially penicillin-resistant *Streptococcus pneumoniae*.

High-performance liquid chromatography (HPLC) with either ultraviolet (UV) or fluorescence detection is the typical analytical method utilized to quantitate fluoroquinolone antibiotics in human biological fluids for use in pharmacokinetic studies. Two exhaustive reviews [1,2] are available that describe the current status of analytical techniques for fluoroquinolone antibiotics. Although numerous pharmacokinetic studies of gatifloxacin have been published in the past several years [3–15], only two published papers [16,17] have described the complete methodology for a validated assay procedure, one employing liquid chromatography with electrospray tandem mass spectrometry, the other using HPLC with fluorescence detection. Several of the gatifloxacin pharmacokinetic studies referenced above describe the assay methodology within the pharmacokinetic paper and all used either fluorescence detection [7–12,18] or a microbiological assay [6,19].

We previously published a method for the separation of six different fluoroquinolone antibiotics and its application to the determination of levofloxacin in human serum for use in a levofloxacin pharmacokinetic study [20]. The analytical method developed in that study was modified for use in determining gatifloxacin concentrations in human serum and urine. The analytical method was validated and used in a gatifloxacin pharmacokinetic study in healthy human volunteers. The study was a drug-interaction study in which gatifloxacin was administered alone and in combination with an enteral feeding product, Ensure. Previous data suggest that enteral feeding products reduce ciprofloxacin and ofloxacin absorption, and as such we required a method that would allow quantitation of gatifloxacin at relatively low serum concentrations [21,22]. Finally, since gatifloxacin is excreted mainly unchanged in the urine it is necessary to estimate renal clearance to fully characterize gatifloxacin pharmacokinetics. Thus, the measurement of gatifloxacin concentrations

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in urine was necessary. The overall aim of the present study was to establish a simple, efficient, reliable, accurate and economical method for the determination of gatifloxacin in human serum and urine.

2. Experimental

2.1. Drug standards, dosage forms and chemicals

Gatifloxacin and ciprofloxacin (internal standard) were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, USA) and Bayer Corporation (West Haven, CT, USA), respectively. Gatifloxacin tablets were manufactured by Bristol-Myers Squibb Company (Princeton, NJ, USA). All chemicals were analytical-reagent grade: sodium phosphate monobasic dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and sodium dodecyl sulfate (SDS) from J.T. Baker (Phillipsburg, NJ, USA); citric acid, acetonitrile, and methanol from EM Science (Gibbstown, NJ, USA); sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$) from VWR Scientific Products (West Chester, PA, USA); tetrabutylammonium acetate (TBAA) from Sigma (St. Louis, MO, USA). The stock solutions of 0.3 M $NaH_2PO_4{\cdot}2H_2O$ and 0.3 M $Na_2HPO_4{\cdot}7H_2O$ were first prepared and then diluted to 0.075 M with water and adjusted to pH 7.5 and 0.5% (mg/ml) SDS was added into the buffer. The displacing reagent consisted of the buffer containing 0.5% SDS with acetonitrile (4:1). All of the above solutions were filtered through a 0.45 μm membrane filter and degassed in an ultrasonic bath for 10 min before use. Water used for preparation of mobile phase solutions, stock solutions, etc., was obtained from a Barnstead, Nanopure Infinity water purification system (Barnstead/Thermolyne, Dubuque, IA, USA). The 47 mm 0.45 μm membrane filter was from Alltech (Alltech Associates Inc., Deerfield, IL, USA). Stock solutions were prepared from gatifloxacin sesquihydrate powder (916 mg gatifloxacin/g powder pure free base) by dissolving the equivalent of gatifloxacin 5 mg in 50 ml of water and 125 mg in 25 ml of acetonitrile for serum and urine assay stock solutions, respectively. Subsequent stock solutions were prepared by serial dilution with deionized water and stored at −20 °C until assay. The standard and control gatifloxacin samples were freshly prepared daily by spiking blank serum and urine with the gatifloxacin stock solutions. The drug free human serum was obtained from Valley Biomedical Inc. (Winchester, VA, USA) and drug free human urine was obtained from healthy subjects. All samples were stored at −70 °C and protected from light until assayed.

2.2. Apparatus

Chromatography was performed on HPLC equipment consisting of a 118 solvent module, a 166 UV detector, and a 507e autosampler (Beckman Instrument Inc., Fullerton, CA, USA). Analytical separation and guard columns were packed with 5 μm Adsorbosphere HS C18 silica stationary phase (Alltech Associates Inc., Deerfield, IL, USA). The dimension of the separation column was 250 mm length × 4.6 mm i.d. with 5 μm particle size. The guard column cartridge was 7.5 mm length × 4.6 mm i.d. with 5 μm particles of identical chemistry to the separation column. Prefilter elements were 4.0 mm diameter with 2 μm particles. Data and chromatograms were collected using Gold Nouveau software (Beckman Instrument Inc., Fullerton, CA, USA). The pH of the solutions was adjusted with a Corning pH/ion meter 450 (Corning Incorporated, Corning, NY, USA). Human serum samples were processed using an Amicon Centrifree micropartition device (Millipore Corporation, Bedford, MA, USA).

2.3. Chromatographic conditions

The mobile phase consisted of 10 mM SDS, 10 mM TBAA, and 25 mM citric acid with 50% acetonitrile in deionized water. Prior to use, the mobile phase was filtered through a 0.45 μm filter and degassed by sonication. Between the sample injections, the injection needle was washed with 70% methanol. The mobile phase was pumped through the system at a rate of 1 ml/min. All experiments were carried out at ambient temperature at approximately 23 °C. The UV detector wavelength was set at 293 nm.

2.4. Sample preparation—serum assay

The blank human serum, calibrator stock solutions, quality control stock solutions, and unknown samples were thawed and vortexed for 30 s. Calibrators and quality controls were freshly prepared by adding 50 μl of each stock solution to 450 μl of blank human serum and vortexing for 30 s. 450 μl of displacing reagent (80% [0.5 mg/ml SDS in 75 mM phosphate at pH 7.5]: 20% acetonitrile) and 50 μl of internal standard (30 μg/ml ciprofloxacin) were added to 500 μl of sample and vortexed for 30 s. Each sample was transferred to an Amicon Centrifree micropartition device and centrifuged at 1500 × *g* for 30 min. Two-hundred microliters of each ultrafiltrate was transferred to a vial for HPLC analysis and the autosampler was programmed to inject 20 μl.

2.5. Calibration standards—serum assay

Calibration curves were produced by injecting processed samples prepared from serum stocks that were spiked to gatifloxacin concentrations of 10,000, 5000, 2000, 1000, 500, 200 and 100 ng/ml. The linear equation describing the relationship between gatifloxacin concentration and the peak area ratio (gatifloxacin to internal standard) was determined

using weighted linear regression analysis, with the weighting method of the squared reciprocal of the concentration for each standard. The coefficient of determination (regression sum of squared residuals/total sum of squared residuals) and visual inspection of the residuals were used as estimates of goodness-of-fit. The final decision on the suitability of the daily calibration curves were made based on the control sample results.

Within-day variability and precision were determined using quality control samples made by spiking blank serum to concentrations of 6000, 1500, 375 and 150 ng/ml. Six processed samples were injected at each control concentration on 1 day. Between-day variability and precision were evaluated by injecting duplicate processed samples at each control concentration and LLOQ each day for 6 days. Control sample concentrations were determined from the standard curve run on each day of analysis. Statistics for the inter-assay study were calculated by using the mean of the replicates for each control concentration. Precision was expressed as the percent coefficient of variation. Accuracy was expressed as the ratio of the mean observed to theoretical concentration as: accuracy (%) = 100 × (predicted concentration/nominal concentration).

2.6. Sample preparation—urine assay

The blank human urine, calibrator, quality control and unknown samples were thawed and vortexed for 30 s. Fifty microliters of sample were diluted with 1000 μl of mobile phase. Fifty microliters of internal standard (30 μg/ml ciprofloxacin) were added to the sample, vortexed for 30 s, and transferred to a vial for HPLC analysis with the autosampler programmed to inject 20 μl.

2.7. Calibration standards—urine assay

Calibration curves were produced by injecting diluted samples prepared from blank urine stocks that were spiked to gatifloxacin concentrations of 150, 50, 10, 5, 2 and 1 μg/ml. Analyses were performed as described in the serum section above.

Within-day variability and precision were determined using quality control samples made by spiking blank urine to concentrations of 100, 37.5 and 3.75 μg/ml. Six samples were injected at each control concentration on 1 day. Between-day variability and precision were evaluated by injecting one processed sample at each control concentration LLOQ each day for 6 days. Control sample concentrations were determined from the standard curve run on each day of analysis. Statistics for the inter-assay study were calculated by using the mean of the replicates for each run (i.e. six runs for each quality control sample). Precision was expressed as the percent coefficient of variation. Accuracy was expressed as the ratio of the mean observed to theoretical concentration as: accuracy (%) = 100 × (predicted concentration/nominal concentration).

2.8. Application of method to a gatifloxacin pharmacokinetic study

Subjects were caffeine and alcohol free for at least 12 h before and during each study day, and avoided all medications for at least 24 h before and during each study day. Each subject received 400 mg gatifloxacin orally alone and 400 mg gatifloxacin orally with an enteral feeding product (Ensure, Abbott Laboratories, Columbus, OH). Blood samples were obtained at 0 h (baseline), and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h after the oral dose. All samples were obtained into non-heparinized evacuated blood collection tubes, allowed to clot and centrifuged at 1500 × *g* for 15 min. After serum separation, all samples were stored at −70 °C until analysis. Urine was collected from each volunteer at the following times after the gatifloxacin dose: 0–24 and 24–48 h. The volume was recorded and a 5 ml sample was collected and stored at −70 °C until analysis. The concentrations of gatifloxacin in serum and urine were determined based on the daily calibration curves. During the analysis of unknown samples from the pharmacokinetic study, the method validation was continued. Acceptance of assay results was determined by monitoring the predicted calibrators and quality control results.

3. Results and discussion

3.1. Selectivity and chromatography

Representative chromatograms are illustrated in Figs. 1 and 2. These chromatograms include a processed blank sample, processed calibrator sample spiked with gatifloxacin and internal standard, and processed subject sample spiked with internal standard in both serum and urine. As is illustrated in each of these chromatograms, the retention times of the ciprofloxacin (internal standard) and gatifloxacin are approximately 5.5 and 6.3 min, respectively. The chromatograms show that ciprofloxacin and gatifloxacin are completely resolved from one another. No interferences were seen in any individual study subject's baseline drug-free serum or urine (representative subject shown in Figs. 1 and 2).

3.2. Linearity

The serum calibration curve was constructed of seven calibrators (100–10,000 ng/ml). The calibration curve was linear over the specified range. The urine calibration curve was constructed of six calibrators (1–150 μg/ml) and was linear over that range. The mean ± S.D. of the slope and intercept of the serum were 4.7E−04 ± 5.3E−05 and −4.7E−03 ± 1.1E−02, respectively. As for the urine, the mean ± S.D. of the slope and intercept were respectively 4.9E−05 ± 8.0E−07 and 8.0E−07 ± 5.7E−03. The coefficient of determination was >0.995 on all calibration curves in serum and urine.

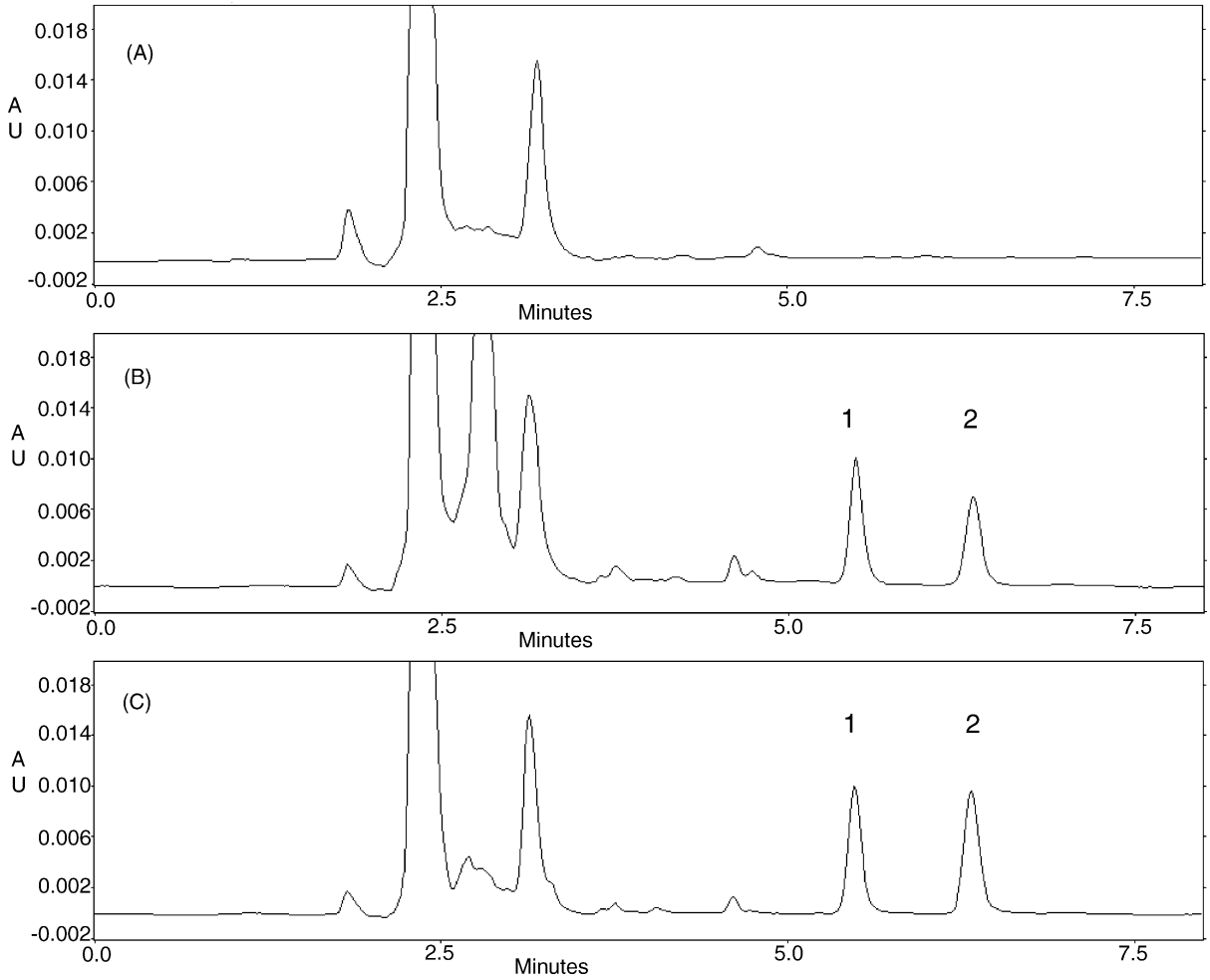

Fig. 1. Chromatograms of blank subject serum sample during gatifloxacin pharmacokinetic study (Panel A), calibrator serum sample spiked with 1500 ng/ml gatifloxacin (found 1504 ng/ml) and internal standard (ciprofloxacin) (Panel B), processed subject serum sample during gatifloxacin pharmacokinetic study (found gatifloxacin concentration 2040 ng/ml) spiked with internal standard (ciprofloxacin) (Panel C). Peak 1: ciprofloxacin (internal standard); Peak 2: gatifloxacin. Detection was by ultraviolet detection as described in the methods.

3.3. Precision and accuracy

The minimum quantifiable serum concentration was 100 ng/ml and the inter-assay CV (precision) and accuracy at this concentration was 5.5 and 92.2%, respectively (six replicate days). The minimum quantifiable urine concentration was 1 μg/ml. The inter-assay CV (precision) and accuracy at this concentration was 8.9 and 100.3%, respectively

Table 1
Intraday and interday precision and accuracy of gatifloxacin in human serum by UV detection

Nominal concentration (ng/ml)	Predicted concentrations (ng/ml)	Precision CV (%)	Accuracy (%)
Interday precision and accuracy ($n = 6$ days of replicate samples)			
150	142 ± 5.68	4.01	94.4
375	362 ± 19.9	5.51	96.2
1500	1490 ± 26.5	1.78	99.2
6000	5970 ± 106	1.77	99.5
Intraday precision and accuracy ($n = 6$ replicate samples)			
150	146 ± 7.41	5.08	97.4
375	356 ± 17.6	4.94	94.8
1500	1496 ± 102	6.82	99.8
6000	5840 ± 115	1.97	97.4

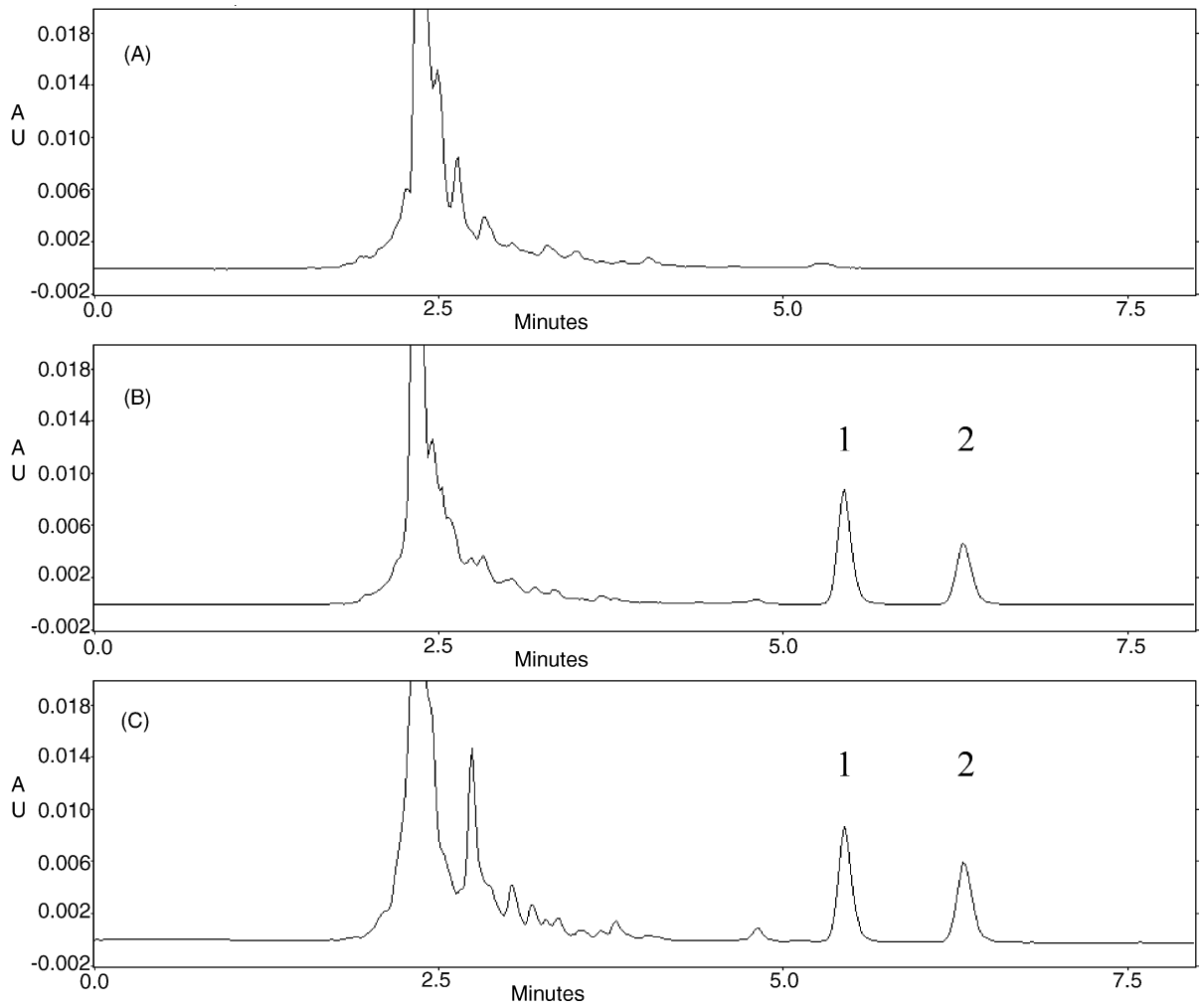

Fig. 2. Chromatograms of blank subject urine sample during gatifloxacin pharmacokinetic study (Panel A), calibrator urine sample spiked with 10 ng/ml gatifloxacin (determined gatifloxacin concentration 9.7 ng/ml) and internal standard (ciprofloxacin) (Panel B), processed subject urine sample during gatifloxacin pharmacokinetic study (determined gatifloxacin concentration 12.6 ng/ml) spiked with internal standard (ciprofloxacin) (Panel C). Peak 1: ciprofloxacin (internal standard); Peak 2: gatifloxacin. Detection was by ultraviolet detection as described in the methods.

(six replicate days). The results of the intra-assay (within day) and inter-assay (between-day) study for the serum assay are listed in Table 1. The results of the intra-assay (within day) and inter-assay (between day) study for the urine assay are listed in Table 2. The results indicate that the method is reliable, reproducible and accurate.

3.4. Stability

The stability of gatifloxacin in human serum was investigated using three replicates of low, medium, and high quality control samples. Freshly made quality control samples were analyzed and stored at −20 °C between freeze thaw

Table 2
Intraday and interday precision and accuracy of gatifloxacin in human urine by UV detection

Nominal concentration (μg/ml)	Predicted concentrations (μg/ml)	Precision CV (%)	Accuracy (%)
Interday precision and accuracy ($n = 6$ days of replicate samples)			
3.75	3.86 ± 0.155	4.02	103.0
37.5	38.9 ± 1.41	3.63	103.7
100	104 ± 4.82	4.62	104.4
Intraday precision and accuracy ($n = 6$ replicate samples)			
3.75	3.64 ± 0.073	2.01	97.1
37.5	39.8 ± 1.72	4.31	106.3
100	103 ± 6.21	6.02	103.0

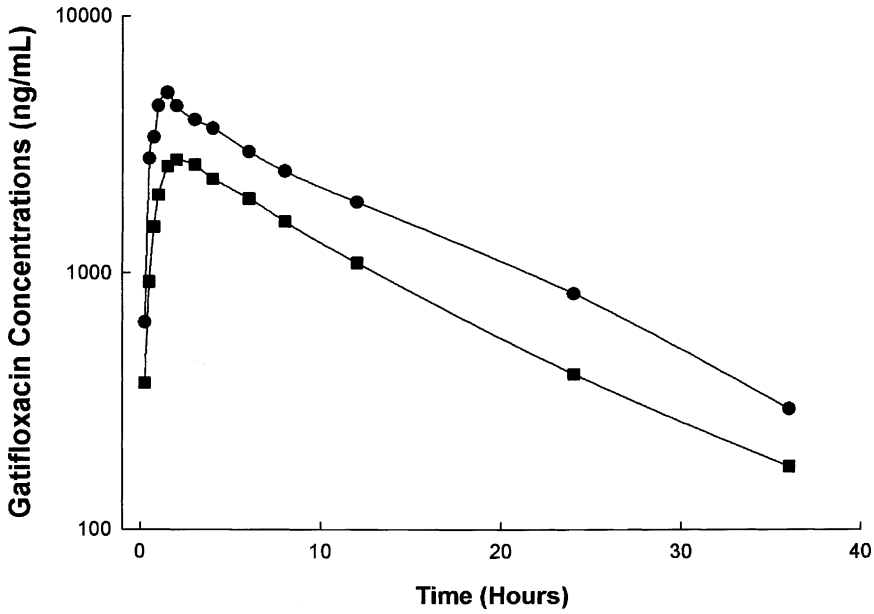

Fig. 3. Individual gatifloxacin serum concentrations vs. time curves in a representative subject. The closed circles (●) represent the observed data points following a 400 mg gatifloxacin oral dose alone or in combination with an enteral feeding product (■).

cycles. The samples were thawed by allowing them to stand at room temperature for approximately 2 h and then refrozen for 24 h periods. Gatifloxacin was considered to be stable in human serum before and after three freeze thaw cycles at 0.15, 1.5, and 6.0 μg/ml with mean (±S.D.) recoveries of 99.2% (3.9), 107.1% (2.1), and 109.6% (4.4) of gatifloxacin concentration in freshly prepared samples, respectively. Additionally, the mean (±S.D.) recovery obtained from three injections of internal standard (20 μg/ml) in mobile phase after three freeze thaw cycles was 106.6% (2.3). Gatifloxacin was stable at room temperature for 24 h (>98% of nominal concentration).

3.5. "Extraction" from human serum

The extraction efficiency was determined by comparing peak areas of directly injected standards in the mobile phase and those from drug-free serum spiked with standards and submitted to the sample preparation and extraction procedures ($n = 6$) at concentrations of 200 and 1000 ng/ml. The mean recovery of gatifloxacin in human serum processed by ultrafiltration was 99 and 95%, respectively. The mean recovery of ciprofloxacin (20 μg/ml) in human serum was 92%.

3.6. Method application

The validated method was applied to patient samples in support of a pharmacokinetic study of gatifloxacin in healthy subjects. Representative chromatograms are illustrated in Figs. 1 and 2. A representative serum concentration–time curve after the administration of gatifloxacin is shown in Fig. 3. The mean (±S.D.) concentrations of gatifloxacin in urine from 0 to 24 and 24 to 48 h were 105.96 (59.42) μg/ml and 33.05 (26.57) μg/ml, respectively, when administered alone and 119.64 (79.58) μg/ml and 30.02 (19.89) μg/ml, respectively, when administered with an enteral feeding product.

4. Conclusions

The assay described in this paper is a useful, relatively simple, highly sensitive, precise, accurate and selective HPLC method for determining gatifloxacin concentrations in human serum and urine. The method was applied successfully to a pharmacokinetic study of gatifloxacin in healthy human volunteers.

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References

[1] G. Carlucci, J. Chromatogr. A 812 (1998) 343.
[2] F. Belal, A.A. Al-Majed, A.M. Al-Obaid, Talanta 50 (1999) 765.
[3] I. Lutsar, I.R. Friedland, L. Wubbel, C.C. McCoig, H.S. Jafri, W. Ng, F. Ghaffar, G.H. McCracken Jr., Antimicrob. Agents Chemother. 42 (1998) 2650.
[4] R. Wise, J.M. Andrews, J.P. Ashby, J. Marshall, J. Antimicrob. Chemother. 44 (1999) 701.

[5] D.M. Grasela, F.P. LaCreta, G.D. Kollia, D.M. Randall, H.D. Uderman, Pharmacotherapy 20 (2000) 330.
[6] D. Honeybourne, D. Banerjee, J. Andrews, R. Wise, J. Antimicrob. Chemother. 48 (2001) 63.
[7] C.K. Naber, M. Steghafner, M. Kinzig-Schippers, C. Sauber, F. Sorgel, H.J. Stahlberg, K.G. Naber, Antimicrob. Agents Chemother. 45 (2001) 293.
[8] A. Lubasch, I. Keller, K. Borner, P. Koeppe, H. Lode, Antimicrob. Agents Chemother. 44 (2000) 2600.
[9] F.P. LaCreta, S. Kaul, G.D. Kollia, G. Duncan, D.M. Randall, D.M. Grasela, Pharmacotherapy 20 (2000) 59S.
[10] F.P. LaCreta, G.D. Kollia, G. Duncan, D. Behr, D.M. Grasela, Pharmacotherapy 20 (2000) 67S.
[11] S. Lober, S. Ziege, M. Rau, G. Schreiber, A. Mignot, P. Koeppe, H. Lode, Antimicrob. Agents Chemother. 43 (1999) 1067.
[12] M. Nakashima, T. Uematsu, K. Kosuge, H. Kusajima, T. Ooie, Y. Masuda, R. Ishida, H. Uchida, Antimicrob. Agents Chemother. 39 (1995) 2635.
[13] A. Mignot, M. Guillaume, M. Brault, V. Gualano, L. Millerioux, K. Gohler, H.J. Stahlberg, Chemotherapy 48 (2002) 116.
[14] A. Mignot, M. Guillaume, K. Gohler, H.J. Stahlberg, Chemotherapy 48 (2002) 111.
[15] A.W. Wallace, J.M. Victory, G.W. Amsden, J. Clin. Pharmacol. 43 (2003) 92.
[16] K. Vishwanathan, M.G. Bartlett, J.T. Stewart, Rapid Commun. Mass Spectrom. 15 (2001) 915.
[17] K. Borner, H. Hartwig, H. Lode, Chromatographia 52 (2000) S105.
[18] D.A. Gajjar, F.P. LaCreta, H.D. Uderman, G.D. Kollia, G. Duncan, M.J. Birkhofer, D.M. Grasela, Pharmacotherapy 20 (2000) 49S.
[19] A. Trampuz, G. Laifer, M. Wenk, Z. Rajacic, W. Zimmerli, Antimicrob. Agents Chemother. 46 (2002) 3630.
[20] H. Liang, M.B. Kays, K.M. Sowinski, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 772 (2002) 53.
[21] B.A. Mueller, D.G. Brierton, S.R. Abel, L. Bowman, Antimicrob. Agents Chemother. 38 (1994) 2101.
[22] O. Mimoz, V. Binter, A. Jacolot, A. Edouard, M. Tod, O. Petitjean, K. Samii, Intensive Care Med. 24 (1998) 1047.