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Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 205–210
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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High performance liquid chromatography for quantification of gatifloxacin in rat plasma following automated on-line solid phase extraction

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Received 18 December 2006; received in revised form 16 February 2007; accepted 21 February 2007
Available online 25 February 2007

Abstract

An automated system using on-line solid phase extraction and HPLC with fluorimetric detection was developed and validated for quantification of gatifloxacin in rat plasma. The extraction was carried out using C_{18} cartridges (BondElut), with a high extraction yield. After washing, gatifloxacin was eluted from the cartridge with mobile phase onto a C_{18} HPLC column. The mobile phase consisted of a mixture of phosphoric acid (2.5 mM), methanol, acetonitrile and triethylamine (64.8:15:20:0.2, v/v/v/v, apparent $pH_{app.}$ 2.8). All samples and standard solutions were chromatographed at 28 °C. The method developed was selective and linear for drug concentrations ranging between 20 and 600 ng/ml. Gatifloxacin recovery ranged from 95.6 to 99.7%, and the limit of quantification was 20 ng/ml. The intra and inter-assay accuracy were up to 94.3%. The precision determined not exceed 5.8% of the CV. High extraction yield up to 95% was obtained. Drug stability in plasma was shown in freezer at −20 °C up to 1 month, after three freeze-thaw cycles and for 24 h in the autosampler after processing. The assay has been successfully applied to measure gatifloxacin plasma concentrations in pharmacokinetic study in rats.

Keywords: Gatifloxacin; On-line solid phase extraction; HPLC; Fluorescence; Validation

1. Introduction

The fluoroquinolones have emerged as one of the most important class of antibiotics and comprise a relatively large and constantly expanding group of substances. Gatifloxacin ((+)-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinoline-carboxyl acid sesquihydrate) (Fig. 1) is a fluoroquinolone with a methoxy side chain at the C-8 position (C8-OMe) which presents a broad spectrum of antimicrobial activity and is used for the treatment of infections of the respiratory and genitourinary tracts [1]. Similar to several other fluoroquinolones, gatifloxacin has enhanced potency against Gram-positive cocci, including multiple-drug-resistant *Streptococcus pneumoniae* isolates [2].

Usually, liquid chromatography (LC) methods with UV and fluorescence detection have been published for the determination of gatifloxacin in biological samples. There are several gatifloxacin pharmacokinetic studies published employing fluorescence detection [3–14], but there is no report on the determination of gatifloxacin using HPLC fluorescence detection coupled with solid phase extraction (SPE).

The preparation of biological samples for HPLC analysis, typically by SPE, can be labor-intensive and prone to errors. A direct injection technique eliminates of multiple sample pre-treatment steps and can increase the throughput of many bioanalytical methods. This strategy is important when the analytes are in a complex chemical or biological matrix. Also, automated sample processing reduces potentially dangerous sample handling. On-line analysis improves analytical precision and reduces solvent volumes used since chromatographic pumps meter the flow rates and the volumes of mobile phase passed through both columns. The procedure can also enhance productivity, allowing unattended processing of large numbers of samples using an autosampler and switching platform connected to the chromatograph's controller. Sample volume can vary from less than a microliter to many milliliters, depending on whether micro-scale columns or analytical columns are used [15].

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doi:10.1016/j.jpba.2007.02.026

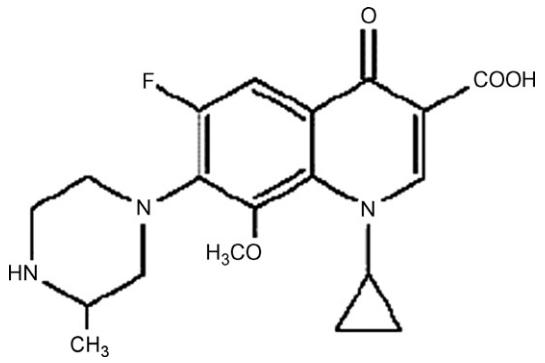

Fig. 1. Molecular structure of gatifloxacin.

In developing an analytical method for pharmacokinetic studies, not only a suitable sensitivity and reproducibility need to be achieved but also labour-saving factors must be considered due to the need of quantifying a large number of samples [16].

The main disadvantage of chromatographic methods is the need for sample preparation, which is the crucial first step in the analysis of biological samples. Different methods are currently applied to prepare biological samples for chromatographic analysis because they cannot be used *per se*. Liquid–liquid extraction (LLE), widely employed, is a tedious, solvent and time-consuming sample preparation. SPE is a consolidated technique for biological samples and it has become more attractive lately because a number of different sorbents are available for both on-line and off-line modes.

Viewing the quantification of gatifloxacin in biological samples a SPE method for sample clean up followed by an on-line reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection is reported in this article. The method has been validated according to published FDA-guidelines [17].

2. Experimental

2.1. Chemicals and reagents

Gatifloxacin (AM-1155) was a gift from Bristol-Myers Squibb. Norfloxacin (internal standard) was obtained from Delaware® (Porto Alegre, Brazil). Methanol and acetonitrile (HPLC grade) and triethylamine and phosphoric acid were purchased from Merck®. Distilled water was purified by a Milli-Q system (Millipore®).

2.2. Preparation of stock and sample solutions

A standard stock solution of gatifloxacin was prepared in methanol with final concentration of 800 μg/ml. Appropriate dilutions of the stock solution were made with purified water pH 2.8 adjusted with phosphoric acid. These dilutions were used to spike plasma for the preparation of calibration curves. The standard stock solutions were prepared in duplicate from separate weightings: one set was used to prepare the calibration curve samples and the other, the quality control (QC) samples. A standard stock solution of norfloxacin (internal standard) was prepared in methanol to produce concentration of 800 μg/ml. For the calibration curve, the final concentrations of gatifloxacin in rat plasma were 20, 35, 75, 100, 200, 400 and 600 ng/ml with 266 ng/ml of norfloxacin. The standard stock solutions, calibration samples and quality controls were freshly prepared daily during ongoing analysis. Standards for the recovery study were prepared by dilution of gatifloxacin stock solution with purified water pH 2.8 adjusted with phosphoric acid. QC samples were prepared at concentrations of 60, 250 and 500 ng/ml and were used for validating the analytical method.

2.3. On-line SPE system and HPLC conditions

Prospekt 2™ (Spark Holland, Emmen, The Netherlands) on-line SPE system consisted of the following modules: automated cartridge exchange (ACE) module for disposable cartridge exchange, high-pressure dispenser (HPD) module for handling of solvents and an autosampler Triathlon. Prospekt 2™ was controlled using SparkLink™ v.2.01. SPE cartridges used consisted of BondElut C_{18}, 10 mm × 2 mm i.d., 40–90 μm particles.

HPLC apparatus consisted of a Shimadzu system equipped with LC-10AD VP pump with a low-pressure gradient flow control valve FCV-10AL VP, SCL-10A VP system controller, SIL-10AD VP autoinjector with 100 μl loop, CTO-10A VP column oven, RF-10AXL detector and a DGU-14A degasser. Shimadzu CLASS-VP software (Version 6.12) was used for data aQCuisition and mathematical calculations. The chromatography was performed on a Shimadzu Shim-Pack CLC-ODS (M) HPLC column (4.6 mm i.d. × 250) with 5 μm particle diameter preceded by a guard column (Nova-Pack C_{18}, 4 μm particle size). All samples and standard solutions were chromatographed at 28 °C using a mixture of phosphoric acid (2.5 mM), methanol, acetonitrile and triethylamine (64.8:15:20:0.2, v/v/v/v) as mobile phase. The apparent mobile phase $pH_{app.}$ was adjusted at 2.8 with phosphoric acid. The sample injection volume was 30 μl. The fluorescence detector was set at excitation wavelength of 295 nm and emission wavelength of 480 nm. The ratio of peak area of analyte to internal standard was used for the quantitation of plasma samples.

2.4. Sample preparation by solid phase extraction

To prepare the calibration curves and QC samples, to each 100 μl of plasma were added 10 μl of gatifloxacin, at different concentrations in order to obtain the final desired plasma concentrations, and 10 μl of internal standard and the mixture was vortexed. The same procedure was performed with the plasma samples for the pharmacokinetic study, except for drug spiking.

The injection and the extraction were completely automated using Prospekt 2™ system and the method developed consisted of the following steps: the cartridge was solvated with 1 ml of methanol at 3 ml/min and equilibrated with 1.5 ml of water at the same flow rate. Following plasma sample injection into the cartridge it was eluted with 1 ml of water at 1 ml/min to retain gatifloxacin and norfloxacin and eliminate plasma proteins. The cartridge was then washed with 2 ml of water and 1.5 ml of

5% methanol at 1 ml/min and eluted with the mobile phase at 1 ml/min. After drugs elution the cartridge was washed with 2 ml of methanol, 1.5 ml of water and 1 ml of 50% methanol. The cartridge was automatically changed for a new one, and a new cycle started. After each injection, the autosampler was programmed to perform a needle wash with 300 μl of 30% methanol.

2.5. Calibration curve and method validation

The method analytical performance was obtained by evaluation of the following parameters: calibration curve linearity, QC intra-day and inter-day precision and accuracy, lower limit of quantitation, specificity and recovery [17].

In order to determine the linearity of the method, three calibration curves with seven calibration points ranging from 20 to 600 ng/ml were prepared in triplicate and analyzed on 2 consecutive days. Daily calibration curves were constructed using the ratios of peak areas of gatifloxacin and the internal standard against nominal concentrations. Linear regression analysis of the data from calibration curves gave slope, intercept and correlation coefficients, which were used to determine the concentration of each analyte in the quality control samples. The lowest concentration level giving a chromatographic response with acceptable coefficient of variation was defined as the lower limit of quantification (LLOQ).

To determine precision and accuracy, quality control samples of gatifloxacin were prepared in triplicate at three concentration levels (60, 250 and 500 ng/ml) and analyzed using the procedure outlined above. Precision was reported as %CV of the estimated concentrations and accuracy was determined by comparing the measured concentrations of the plasma samples to the nominal concentration spiked into them.

The method recovery was evaluated by comparing the peak area of extracted QC plasma samples to the peak area obtained with standard solutions directly injected into the LC system. The selectivity of the assay was investigated by processing and analyzing blank samples prepared from six independent lots of control plasma as well as by analyzing plasma samples obtained after oral administration of the compound to Wistar rats. The blanks and the animal samples were surveyed for interfering peaks.

2.6. Preliminary stability studies

The short-term stability of gatifloxacin and the internal standard in plasma was studied under two experimental conditions: during three freeze-thaw cycles and after storage at the autosampler carousel at room temperature for 24 h. Long-term stability in serum was assessed after 1 month storage at −20 °C. Stability was evaluated by comparing measured concentrations before and after storage. The analyses were carried out in triplicate.

2.7. Pharmacokinetic study

The protocol used for gatifloxacin pharmacokinetic evaluation was previously approved by the Ethics in Research Committee of Universidade Federal do Rio Grande do Sul—UFRGS (#2005413).

A pilot study was carried out to demonstrate the applicability of the method developed. Male Wistar rats (250–300 g) were housed in stable conditions of humidity (60 ± 5%), temperature (21 ± 3 °C) and 12 h light–12 h dark cycle. Water was freely available and they were deprived of rat standard chow 12 h before drug administration.

Gatifloxacin was dissolved in a 0.9% sterile saline solution and administered orally as a single dose of 6 mg/kg body weight ($n = 4$). This dose corresponds to the human daily dose (400 mg) assuming a 70 kg BW. Blood samples (200 μl) were colleted into sodium heparinized tubes by the lateral tail vein, before drug administration (time 0) and at 0.33, 0.66, 1, 1.5, 2, 4, 6, 8 and 10 h after administration. Blood cells were removed by centrifugation and the separated plasma was stored at −20 °C until assayed. Pharmacokinetic parameters were determined by non-compartmental approach. The elimination rate constant (k_{el}) was calculated by log-linear regression of the gatifloxacin concentration during the elimination phase and the half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. Gatifloxacin peak plasma concentration (C_{max}) and time of peak (t_{max}) were obtained by visual inspection of the concentration–time profiles. The area under the plasma concentration versus time curve (AUC_{0-t}) from time zero to the time of last concentration measured (C_{last}) was calculated by the log-linear trapezoidal rule. The AUC zero to infinity ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/k_{el}.

3. Results and discussion

The HPLC method developed provided a simple and fast procedure for the quantification of gatifloxacin in biological samples. In preliminary studies, the removal of interfering plasma components was attempted by LLE and no success was achieved. The largest recoveries and the cleanest extraction procedure were achieved by using SPE method. The BondElut C_{18} cartridges provided better recovery and resolution between the analyte and the internal standard investigated than others cartridges previously evaluated in pilot studies.

Preliminary studies with different mobile phase combinations of phosphate buffer, methanol and acetonitrile were considered. Different pH of the mixture were evaluated and the optimum apparent $pH_{app.}$ was 2.8. Chromatographic conditions were based on isocratic separation on a reverse phase column, preceded by a guard-column.

3.1. Lower limit of quantification

The LLOQ of the method developed was 20 ng/ml, which was the lowest concentration of analyte in the method development. This concentration presented accuracy not deviate by more than 20% of the actual value and precision not exceed 20% of the CV under the stated experimental, according to the FDA Guidelines [17].

Table 1
Intra- and inter-assay precision of the HPLC assay ($n = 6$)

Nominal concentration (ng/ml)	Day	Measured concentration[a] (ng/ml)	Accuracy (%)	CV[b] (%)
Intra-assay				
60	1	59.5 ± 3.4	99.2	5.7
250	1	244.8 ± 6.1	97.9	2.5
500	1	486.1 ± 25.4	97.2	5.2
60	2	62.1 ± 3.0	102.2	4.8
250	2	235.7 ± 5.7	94.3	2.4
500	2	527.3 ± 25.3	105.5	4.8
Inter-assay				
60		60.8 ± 2.9	100.7	4.8
250		240.3 ± 6.6	96.1	2.8
500		506.7 ± 29.2	101.3	5.8

[a] Mean ± S.D.
[b] CV: coefficient of variation.

3.2. Linearity

Plasma samples spiked with different concentration levels of gatifloxacin were analyzed using the optimum SPE conditions. The linearity of the standard curve was checked in six different runs after calculating individual slopes and intercepts of each individual curve. A linear response was observed over the examined concentration range (20–600 ng/ml). The coefficients of determination (R^2) obtained were 0.9998 ($y = 0.0104x + 0.2184$), 0.9996 ($y = 0.0097x + 0.3620$), 0.9997 ($y = 0.0107x + 0.2216$), 0.9997 ($y = 0.0101x + 0.2917$), 0.9997 ($y = 0.0090x + 0.3564$) and 0.9995 ($y = 0.0099x + 0.2435$). The correlation coefficient was ≥0.9995 on all calibration curves in plasma. On average, the slopes of the calibration curves obtained in different analysis and in different days did not vary considerably, and the intercepts obtained were near to theoretical zero value, demonstrating good constancy of the measuring system.

3.3. Precision, accuracy and extraction efficiency

Intra-assay and inter-assay precision and accuracy were performed. The results are reported in Table 1. The intra-assay CV% and inter-assay CV% were below 5.7 and 5.8, respectively. Acceptable accuracy was achieved for all concentrations investigated and these values are adequate for biological samples. [17].

The recoveries from plasma and aqueous standard solution of gatifloxacin and internal standard were evaluated to test the efficiency and reproducibility of the plasma cleaning up procedure by SPE. The recovery of gatifloxacin ranged from 95.6 to 99.7% and it was independent of the drug concentration investigated, as reported in Table 2. The recovery of internal standard was 101.1%.

3.4. Selectivity

When analyzing drugs from biological fluids using SPE, the adequate selection of the convenient extraction conditions (sample pH, composition of the washing and elution solvents, and the nature of the sorbent material) provide the cleanest samples for the chromatographic analysis and is an important factor for the selectivity of the entire analytical method. The correct selection of SPE washing and elution solvents provided very clean samples and an adequate selectivity for the analytical method developed. The optimum conditions were obtained using BondElut C_{18} cartridges.

Table 2
Extraction efficiency of gatifloxacin and internal standard from rat plasma using SPE-HPLC method

Nominal concentration (ng/ml)	Mean recovery (%)	S.D.	Range (ng/ml)
Gatifloxacin ($n = 3$)			
60	97.4	2.5	57.2–60.1
250	95.6	2.5	234.7–246.1
500	99.7	1.5	491.1–506.1
Norfloxacin[a] ($n = 9$)			
266	101.1		

[a] Internal standard.

As is illustrated in the chromatograms showed in Fig. 2, the retention times of norfloxacin (internal standard) and gatifloxacin were approximately 4.2 and 5.8 min, respectively. No endogenous peaks or metabolites that would interfere with the quantification of gatifloxacin and internal standard were observed for different plasma sources and after drug administration.

3.5. Stability

The concentrations of gatifloxacin and norfloxacin in plasma samples left in the autosampler carousel after 24 h at room temperature did not reduce significantly in relation to time zero. In this condition, gatifloxacin standard sample concentrations 20 and 600 ng/ml dropped 1.9 and 1.7%, respectively. The corresponding internal standard concentration decreased 2.9 and 1.4%. These results showed the stability of both drugs in processed plasma samples during analysis time.

The samples were stable in plasma at −20 °C for at least 1 month, with recovery up to 98%. Regarding the freeze-thaw cycles the recovery decreased 6% after the third cycle, but this reduction on drug concentration was not statistically significant ($p > 0.05$).

3.6. Pharmacokinetic study

The SPE-HPLC method developed was employed to determine the pharmacokinetic profile of gatifloxacin after single oral dose of 6 mg/kg to Wistar rats. Gatifloxacin average concentration versus time profile is shown in Fig. 3. The peak plasma concentration was 409.5 ± 97.0 ng/ml and it took place at 1.5 ± 0.5 h. The half-life of the elimination phase was 2.5 ± 0.4 h. The $AUC_{0-\infty}$ was 1974 ± 386 ng ml h^{-1} and extrapolated AUC ($AUC_{t-\infty}$) was 156 ± 36, less than 10% of the total AUC, demonstrating that the sensitivity of the method developed is sufficient to allow quantifying the drug in rat plasma

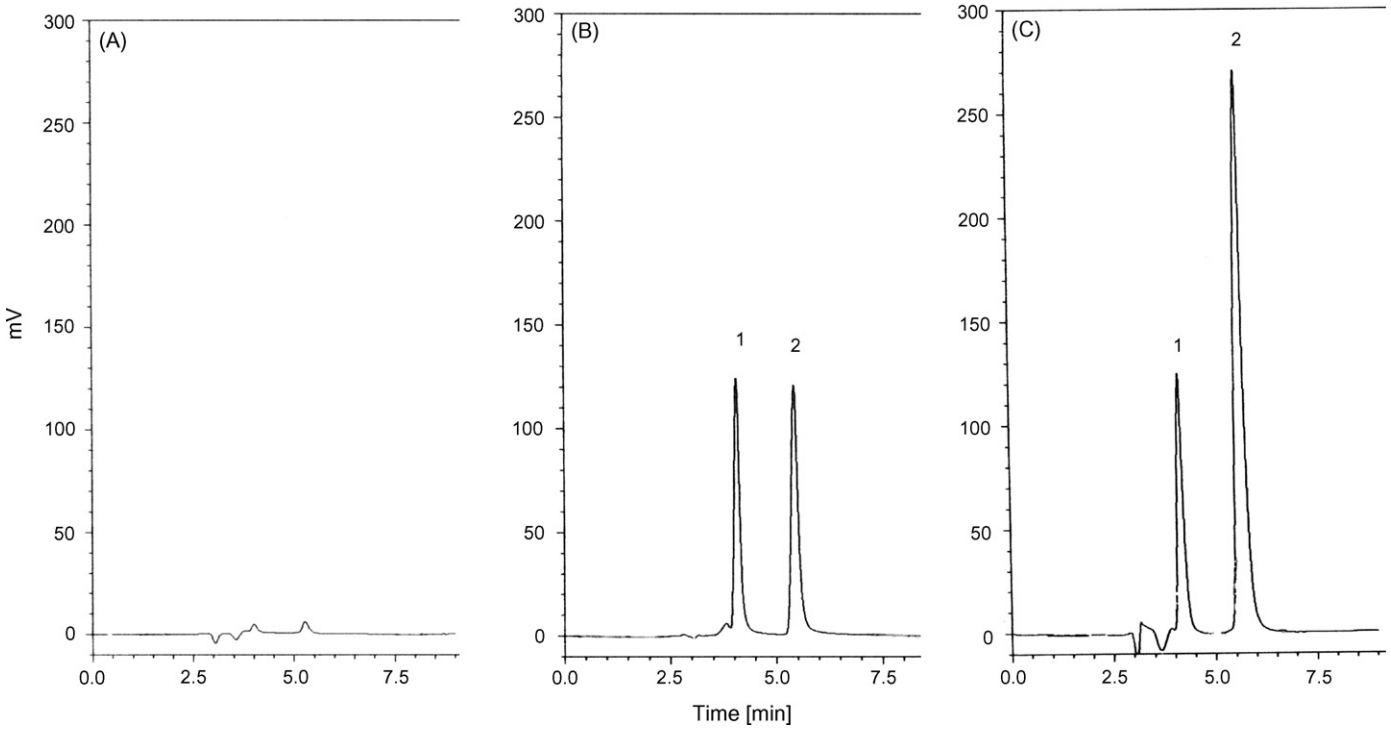

Fig. 2. Representative HPLC chromatograms of the method: (A) blank rat plasma; (B) rat plasma spiked with norfloxacin 266 ng/ml (internal standard) (1) and gatifloxacin 100 ng/ml (2); (C) 4 h plasma sample (214 ng/ml) from rat dosed with gatifloxacin 6 mg/kg p.o. and internal standard. Norfloxacin and gatifloxacin retention times were 4.2 and 5.8 min, respectively.

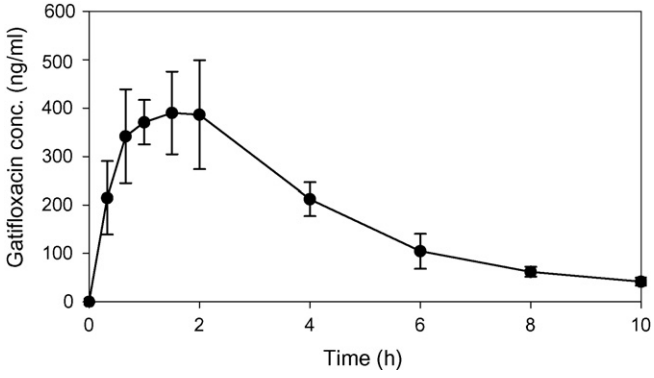

Fig. 3. Mean plasma concentration–time profile of gatifloxacin after oral administration of 6 mg/kg to four Wistar rats. Each point represents mean ± S.D.

for a period of time long enough to adequately characterize its elimination phase.

4. Conclusions

A bioanalytical method for gatifloxacin quantification in rat plasma has been developed and validated. This method is simple and highly reliable with no interfering peaks from endogenous compounds or metabolites and can be used to determine the drug concentration in pre-clinical pharmacokinetic studies. The method reported advances the analysis of gatifloxacin in plasma by combining a rapid and efficient on-line SPE drug extraction with a specific and sensitive quantification by HPLC with fluorescence detection.

Acknowledgements

The authors are grateful to Brystol-Myers Squibb for providing gatifloxacin sample. This work was financially supported by the Research Foundation of the State of Rio Grande do Sul (FAPERGS/Brazil).

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