Solid Phase Extraction-HPLC Method for Determination and Pharmacokinetic Study of Garenoxacin in Rat Plasma after Oral Administration

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Abstract : A method based on solid phase extraction was developed for the determination of Garenoxacin in rat plasma after oral administration by high-performance liquid chromatography coupled with PDA detection (HPLC–PDA). Variables parameter affecting the solid phase extraction efficiency were evaluated and optimized. Chromatography separation was performed on a THERMO, BDS HYERSIL C18 column (4.6 mm $\times$ 100mm, 5μ) by isocratic elution with PDA detection at 279 nm. The assay was linear over the range of 15- 44 μg/ml and the lower limit of quantification (LLOQ) was 15 μg/ml. The extraction recoveries were more than 77 %, the accuracies were within 3.97%, and the intra- and inter-day precisions were less than 9.36% in all cases. After strict validation, the method indicated good performance in terms of reproducibility, specificity, linearity, precision and accuracy, and it was successfully applied to the pharmacokinetic study of Garenoxacin in rats after oral administration.

Keywords : Solid Phase extraction; Garenoxacin; HPLC–PDA; Pharmacokinetics.

Introduction

Garenoxacin, 1–Cyclopropyl -8- (difluoromethoxy) -7- [(1R)-1-methyl-2, 3-dihydro-1H-isoindol -5-yl]- 4- oxo-1, 4-dihydroquinoline-3-carboxylic acid, is a quinolone antibiotic which exert broad range of antibacterial activity against Gram-negative and Gram-positive pathogens, including certain quinolone-resistant strains.[1] A variety of methods have been developed for determination of Garenoxacin individually or with combination with some other agents in biological samples. This includes, RP-HPLC[2] method for assay, metabolism and deposition study in rats, monkey and dog[3], joint cartilage study in immature rats[4], simultaneous estimation in human urine by HPLC-UV[5] and fluorescence and terbium-sensitized luminescence determination in human urine and plasma[6]. None of these provide simple determination of Garenoxacin in rat plasma and its application in pharmacokinetic assessment of same in rats. Thus, the present study has been undertaken to develop and validate a simple, sensitive, accurate, precise and reproducible bioanalytical HPLC method for estimation of Garenoxacin in rat plasma.
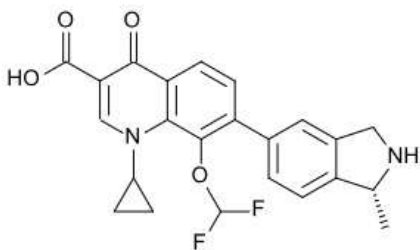
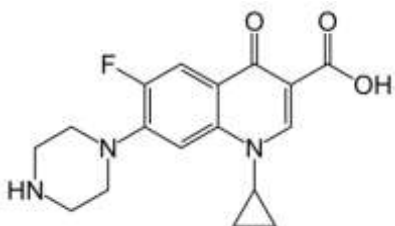**Fig. 1 A: Chemical structure of Garenoxacin**

Fig. 1 B: Chemical structure of Ciprofloxacin

Experimental

Materials and Reagents

Garenoxacin (Fig. 1 A) and Ciprofloxacin (used as an internal standard (IS)) (Fig. 1 B), was obtained from was procured from reputed company and certified to have a purity of 99.8% and 99.9% respectively. O-phosphoric Acid, Potassium Dihydrogen Phosphate, purchased from Merck Specialties Private Limited (Mumbai, India) and these materials were of analytical grade. Acetonitrile (HPLC grade) and Methanol (HPLC grade) was purchased from Merck Specialties Private Limited (Mumbai, India). Milli-Q^{TM} water (Millipore, Milford, MA, USA) was used in completing work.

Instrumentation and Chromatographic Condition

Chromatography was preformed using a Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan) consisted of a LC-20AD quaternary pump, DGU-20A$_5$ degasser for degassing solvent and a Shimadzu SPD-M20A PDA detector. The separation was carried out on a THERMO, BDS HYERSIL C18 column (4.6 mm × 100mm, 5μ). The mobile phase was acetonitrile- 25 mM potassium dihydrogen phosphate (pH 3.3, adjusted with O- phosphoric acid) in ration of 25:75 v/v. The mobile phase was filtered using a 0.2μm Teflon membrane filter (Millipore, Milford, MA, USA) and degassed by sonication prior to use. The flow rate was 1.0 ml/min and UV detection was carried out at wavelength 279 nm.

An Equip-Tronics EQ 621 pH meter (Mumbai, Maharashtra, India), equipped with a combined glass electrode was used for pH adjustment. For solid phase extraction was carried out on Hypersep Retain PEP, (30mg, 1 ml), (Thermo Fisher Scientific, Mumbai) cartridge using Thermo 16 port vacuum manifold (Thermo Fisher Scientific, Mumbai) at negative pressure. The plasma samples were stored at Remi Ultra Low Deep Freezer (Remi Sales and Engineering Ltd., Goregaon, Mumbai) at -80°C and Nitrogen evaporator (Min-evap, Takahe Analytical Instruments, Mumbai) was used for evaporation. A vortex mixer (CM-101 Plus, Remi Sales and Engineering Ltd., Goregaon, Mumbai) was utilized to blend the solution adequately.

Preparation of Standard and Quality Control Standard

A stock solution of Garenoxacin (1000 μg/ml) was prepared in methanol and a series of standard solutions were obtained by further dilution of the stock solution with methanol. A stock solution of Ciprofloxacin (internal standard) was prepared in methanol at 2200 μg/ml and subsequently working solution at a strength of 42 μg/ml. All solutions were stored at 4°C. Calibration standards in plasma were prepared daily by

spiking 10µl of the standard solutions in to 90µl blank rat plasma. The concentrations of Garenoxacin was 50 mg/kg of body weight for oral administration.

The quality control (QC) samples used in the validation and during the pharmacokinetic studies were prepared in the same way as the calibration standard at appropriate concentrations.

Sample Preparation by Solid Phase Extraction Procedure

For the extraction and pre concentration of Garenoxacin, 90 µl of rat plasma sample, 10µl of Garenoxacin and 10µl of internal standard solution (42 µg/ml) were added to a 2.0 ml tapered centrifuge tube and vortexed for 5 min. Above solution was loaded on pre-equilibrated (using methanol) and pre-washed (using water) cartridge which is mounted on vacuum manifold. After loading of sample, cartridge was washed using methanol and subsequently analyte was eluted using methanol. The resultant supernatant was evaporated at 50°C using nitrogen gas stream at pressure of 0.8 to 2.0 psi in nitrogen evaporator. The residue was reconstituted using 500µl mobile phase and vortexed for 5 min and 20µl of reconstituted sample was injected into the HPLC system for analysis.

Method Validation

To determine the linearity of the method, calibration standards were prepared in triplicate and analyzed in three separate analytical runs. Daily calibration curves were constructed using plot of peak area ratio of Garenoxacin and ciprofloxacin versus the concentrations of Garenoxacin. The unknown sample concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve. To determine precision and accuracy, QC samples were prepared at three concentrations levels 18, 32 and 43 µg/ml. Six replicates were analyzed in each of three analytical runs. The precision was evaluated by the relative standard deviation (RSD.); the accuracy was determined by calculating the percentage deviation of the observed concentrations from the nominal concentrations and expressed as relative error (RE). The precision and accuracy were required to be within ±20% for the lower limit of quantification (LLOQ) and within ±15% for other concentrations. Recovery of the extraction procedure was evaluated at low, medium and high concentrations for Garenoxacin, and at 42µg/ml for the IS. It was determined by comparing the mean peak areas (n = 6 at each concentration) obtained from plasma samples spiked before extraction to those from plasma samples spiked after extraction.

To evaluate the stability of Garenoxacin in rat plasma, spiked QC samples of two different concentrations were subjected to three freeze–thaw (-25°C) cycles, the post-preparative stability storage at 4°C for 24 h and the long-term stability storage at -80°C for 20 days were also studied. The bench top stability was assessed at room temperature for 4 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of those prepared freshly. Samples were to be regarded as stable if bias of them were within ±15% of the actual value.

Application of the method and pharmacokinetic study

Male Wister rats (male 180- 250 mg; Female 160–210 mg) was procured from the Animal house of Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, were kept in an environmental controlled breeding room for 7 days before starting the experiment. All procedures involving animals were in accordance with the Regulations and permission for use of them is obtained from Institutional Animal Ethics Committee at Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, MS, India.

Before drug administration, the rats were fasted overnight with free access to water. Garenoxacin suspension in carboxy methyl cellulose was prepared and administrated orally (50 mg/kg). Blood samples (0.5 ml) were collected from the ocular vein into heparinized tube before (0 h) and 0.5, 1, 2, 4, 8, 6, 12 and 24 h after dosing, and then immediately centrifuged at 3500 rpm for 10 min. The plasma samples were transferred into new tubes and stored frozen at -80°C until analysis. Because of a large volume of blood sample was required, 24 (12 males and 12 females) rats were divided into four groups each for male and female rats of three animals each. Blood was collected from each group at five time points. The plasma concentrations of Garenoxacin at different time points were expressed as mean ± S.D., and the mean concentration-time curves were plotted. All the pharmacokinetic data were calculated using the Kinetica 2.0 v statistical software.

Results and Discussion

HPLC-PDA conditions

To optimize the chromatographic conditions, the mobile phase system was investigated. Preliminary studies on different mobile phase combinations of phosphate buffer, methanol and acetonitrile were considered. The pH of the mobile phase was also explored. Mobile phases of pH 6.5 or 4.5 yielded tailing peaks. While the pH of the mobile phase was adjusted to 3.3, the peaks became sharp and symmetric. Based on this, acetonitrile and phosphate buffer were selected. The type and molar concentration of phosphate buffer and the ratio between acetonitrile and phosphate buffer were also optimized in order to achieve good resolution and separation of analytes as well as short run time. It was found that a mixture of acetonitrile and 25mM potassium dihydrogen phosphate (adjusted to pH 3.3 with phosphoric acid) (25:75, v/v) delivered at a flow rate of 1.0 ml/min could achieve our purpose, and was finally adopted. Taking both two compounds into consideration, 279 nm was selected as the detection wavelength in our study.

Method validation

Specificity

The specificity of the method was evaluated by comparing chromatograms obtained from six independent plasma samples from rats, each as a blank and a spiked sample. Typical chromatograms are shown in Fig. 2. The analyte and internal standard were both eluted without any interference from endogenous substances. The retention times for Garenoxacin and IS were 4.157 and 1.632 min, respectively. And under the described chromatographic conditions, no metabolites of Garenoxacin were observed in rat plasma. The results showed that the described HPLC method was selective for the determination of Garenoxacin in rat plasma.
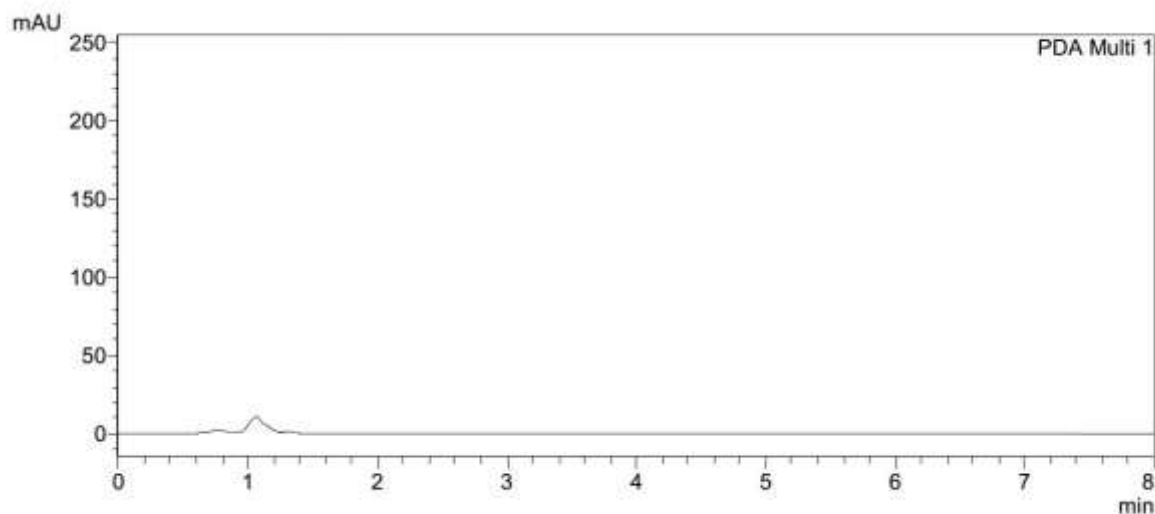

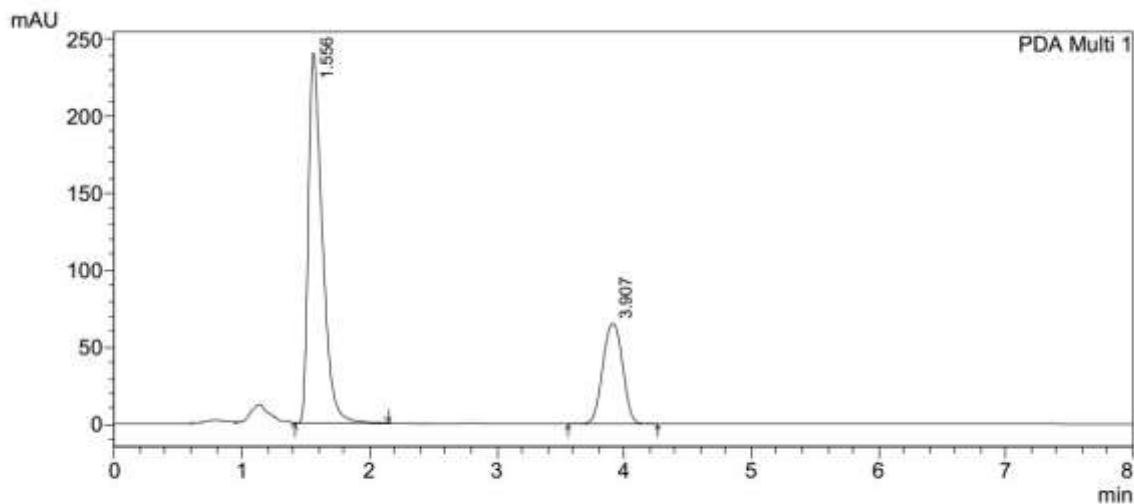**Fig. 2 A: Typical chromatograms of blank plasma.**

Fig. 2 B: Typical chromatograms of Garenoxacin and I.S.

Linearity

The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of Garenoxacin against internal standard versus the nominal concentration (x) of Garenoxacin. The calibration curves were obtained by weighted ($1/x2$) linear regression analysis. The plotted calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration range 15–44 µg/ml for Garenoxacin.

Limit of quantification

The LLOQ for determination of Garenoxacin in rat plasma, defined as the lowest concentration analyzed with an accuracy ≤15% and a precision ≤15%, was found to be 15 µg/ml.

Accuracy and precision

The intra- and inter-day precisions and accuracy results are summarized in Table 1. In this study, the intra- and inter-day precisions were less than 9.36% RSD, and the accuracy was within 3.96% for each QC sample. The obtained values were lower than the limits required for biological sample analysis. These data indicated that the assay was reproducible, accurate and reliable.

Extraction recovery

The results (Table 1) showed that extraction recoveries of Garenoxacin from rat plasma, were 82.65, 77.64, 79.67 and 80.15 % at concentrations of 15, 18, 32 and 42 µg/ml, respectively. For the internal standard (42 µg/ml), the mean extraction recovery was 84.4 %.

Stability

The stability of Garenoxacin in plasma was evaluated by analyzing spiked quality control samples. The stability data was summarized in Table 2, which indicated Garenoxacin was stable under different conditions.

Pharmacokinetic study of Garenoxacin in rats

The method yielded satisfactory results for determination of Garenoxacin in rat plasma and has been successfully applied to the pharmacokinetic studies of Garenoxacin after oral administration to rats. The pharmacokinetic data are listed in Table 3.

Table 1: Precision, accuracy and recovery of Garenoxacin assay in rat plasma

Concentrations (µg/ml)	Precision				Accuracy (RE %)	Recovery Mean (%)
	Intra-day (n=6)		Inter-day (n=6)			
	Mean ± SD (µg/ml)	RSD (%)	Mean ± SD (µg/ml)	Mean ± SD (µg/ml)		
15	2.53	3.06	3.70	5.15	1.96	82.65
18	2.23	2.98	1.86	2.30	2.70	77.64
32	3.15	3.83	1.40	1.90	1.05	79.67
42	9.15	9.35	5.34	5.08	3.96	80.15

Table 2: Stability of Garenoxacin in rat plasma

Stability Parameters		LQC (18 µg/ml)	HQC (42 µg/ml)
Plasma stored at room temperature for 4 h			
	SD	2.08	1.96
	RSD (%)	2.66	2.41
	RE (%)	1.48	1.05
Long-term stability (storage at -80 ^{O}C for 20 days)			
	SD	1.04	1.74
	RSD (%)	2.07	2.21
	RE (%)	0.73	0.58
Freeze and Thaw Stability			
	SD	1.39	1.13
	RSD (%)	1.54	2.35
	RE (%)	1.18	2.07

SD: standard deviation; RSD: relative standard deviation; RE: relative error

RE (%)= 100 X ((mean concentration- nominal concentration)/nominal concentration)

Table 3: Pharmacokinetics data of Garenoxacin in rats

Parameters	Estimate (Mean ± SD (µg/ml)
	Oral administration
$t_{1/2}$ (h)	11.45
T_{max} (h)	4
C_{max} (µg/ml)	6.12
AUC_{0-t} (µg/ml*h)	76.57
$AUC_{0-\infty}$ (µg/ml*h)	98.71

Conclusion

The solid phase extraction technique was applied as an effective method for the extraction and pre-concentration of Garenoxacin from plasma samples. Coupled with HPLC–PDA detection, the method has been proved to be simple, rapid, sensitive, accurate and reliable for assessment of Garenoxacin in biological samples and pharmacokinetic studies for pre-clinical purpose.

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