Bioanalytical method development and validation of garenoxacin mesylate in human plasma by RP-HPLC

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ABSTRACT

A simple, precise, accurate Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed for the estimation of Garenoxacin mesylate in human plasma using Ciprofloxacin Hydrochloride as an internal standard. Chromatographic conditions used are stationary phase Zorbax Eclipse XDB C18 (250x4.6 mm, 5μ), Mobile phase 0.1% orthophosphoric acid and Acetonitrile in the ratio of 50:50 (% v/v) and flow rate was maintained at 1.0 ml/min, detection wavelength was 240 nm, injection volume of 50 μL and the column temperature was set to 30°C. The retention time of Garenoxacin mesylate was found to be 4.0 min. % Coefficient of Variation of the Garenoxacin mesylate was found to be 4.30. % Recovery was obtained as 98.97%. The linearity of the proposed method was established in the concentration range of 0.04 to 4 μg/ml (Correlation Coefficient = 0.999). The lower limit of quantification was 0.04 μg/ml (S/N Ratio 21) which reach the level drug possibly found in human plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range.

INTRODUCTION

Antibiotic resistance is a rising concern and a problem yet to be answered especially for respiratory tract infections. Garenoxacin mesylate 1-Cyclopropyl-8-(difluoromethoxy)-7-[(1R)-1-methyl-2,3-dihydro-1H-isindol-5-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylicacid monomethane sulphonate monohydrate is a newly developed novel desfluoro (6) quinolone in Japan that was further researched and developed by Toyoma Ltd in collaboration with Bristol Meyers Squibb, USA (Agam Vora., 2014). Garenoxacin mesylate with unique Pharmacokinetic profile promises to cover a wide spectrum of organisms commonly encountered in community-acquired infections including Gram-positive, Gram-negative, Atypical & Anaerobic organisms with negligible potential for resistance development (Ince.D et al., 2002; Gajjar.A. D et al.,2003; Takagi et al., 2008; Hori.S., 2011; Kohno.S., 2013). The Extensive review revealed that few analytical methods like UV (Aboli Edlabakar et al., 2016; Sakariya et al., 2015), RP-HPLC (Aboli Edlabakar, 2018; Ashwin Kumar et al., 2017) have been reported for estimation of Garenoxacin mesylate in dosage form and biological fluid (Rajendra et al., 2017). Hence a simple, precise, accurate, sensitive, selective, reproducible
and rapid analytical technique for the estimation of Garenoxacin mesylate, in plasma is developed and validated as per ICH guidelines (ICH Q2B). The Chemical structure of Garenoxacin mesylate is shown in Fig. 1.

![Chemical structure of Garenoxacin mesylate](image)

**Figure 1: Chemical structure of Garenoxacin mesylate**

**MATERIALS AND METHODS**

**Reagents and Chemicals:** Garenoxacin mesylate was obtained as a gift sample from Spectrum Pharma Research Solutions (Hyderabad, India) HPLC grade Acetonitrile, Methanol and Water were acquired from Merck (Mumbai, India). All the chemicals and reagents used were of HPLC grade and purchased from Finar and Merck.

![Chromatogram of extracted Standard Blank](image)

**Figure 2: Chromatogram of extracted Standard Blank**

**Instrumentation and Chromatographic Conditions:** Chromatography was performed using Waters HPLC 2695 System equipped with quaternary pumps, Photo Diode Array detector and Autosampler integrated with Empower 2 Software. The separation was carried out on a Zorbax Eclipse XDB C18 column (250 x 4.6 mm, 5µ). The mobile phase was 0.1% Orthophosphoric acid: Acetonitrile in the ratio of 50:50 (%v/v). The mobile phase was filtered using a membrane filter (0.45 µ) and degassed. The flow rate was maintained at 1.0 ml/min, and the effluent was monitored at 240 nm.

**Preparation of Solution**

**Preparation of Garenoxacin mesylate Stock solution (2 mg/ml):** Accurately weighed and transferred 200 mg of Garenoxacin mesylate into a 100 ml volumetric flask, added 60 ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed (2 mg/ml of Garenoxacin mesylate).

**Preparation of Garenoxacin mesylate Spiking Solutions (0.04 µg/ml to 4 µg/ml):** From the above Garenoxacin mesylate stock solution 0.046ml, 0.092ml, 0.138ml, 1.15ml, 1.725ml, 2.3ml, 3.68ml and 4.6 ml was pipetted out and transferred to 8 individual 10 ml volumetric flask and made up the volume upto the mark with diluent to produce 9.2 µg/ml, 18.4 µg/ml, 27.6 µg/ml, 230 µg/ml, 345 µg/ml, 460 µg/ml, 736 µg/ml and 920 µg/ml.

**Preparation of Calibration and Quality control samples:** Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.04 µg/ml, 0.08 µg/ml, 0.12 µg/ml, 1.0 µg/ml, 1.50 µg/ml, 2.0 µg/ml, 3.2 µg/ml and 4.0 µg/ml.

**Preparation of internal standard Solution (4 µg/ml):** Accurately weighed and transferred 40 mg of Ciprofloxacin Hydrochloride in 100 ml volumetric flask added 60 ml of diluent and sonicated to dissolve and diluted to volume with diluent and mixed (400 µg/ml of Ciprofloxacin Hydrochloride). From the above solution, transferred 4.6 ml into a 10 ml volumetric flask and diluted to volume with diluent and mixed (184 µg/ml of Ciprofloxacin Hydrochloride).

**NOTE:** From the above solution, take 0.05ml (50 µL) of the solution and spiking blank plasma with working stock dilutions of analyses to produce 4 µg/ml internal standard concentration.

**The extraction procedure:** Transferred 250 µL of plasma and 50 µL of internal standard, 10 µL of Garenoxacin mesylate from the spiking solutions into a centrifuging tube and vortex for 15 sec. Added 2 ml of Acetonitrile to the above centrifuge tube and vortex for 2 min. It is centrifuged the above centrifuge tube at 4000 rpm speed for 10 min after the centrifugation collected the clear liquid and filtered the solution through 0.45 µ membrane filter.

**RESULTS AND DISCUSSION**

**Method Development**

Optimization of the chromatographic conditions was performed based on retention time, resolution, asymmetric factor, and peak area obtained for Garenoxacin mesylate. The mobile phase was selected on the basis of its polarity, and different trials were taken. In the Optimised method retention time of Garenoxacin and Ciprofloxacin was found to be 4.0 min and 3.4 min respectively. The developed method is time-saving, and a number of sam-
samples can be estimated in less time. A typical chromatogram of Garenoxacin mesylate obtained by optimized conditions is shown in Fig. 3.

**Bio-analytical Method Validation**

The developed analytical method for the estimation of Garenoxacin mesylate is validated as per ICH Guidelines.

**System Suitability**

System suitability experiment was performed by injecting 6 consecutive injections using aqueous sample equivalent to Medium QC (MQC) concentration. System suitability was performed at the start of the method validation and on each day as a first experiment. The results of the System suitability are given in Table 1.

**Selectivity/Specificity**

To establish the selectivity of the method, possible interference at the retention time of Garenoxacin mesylate and Ciprofloxacin hydrochloride due to endogenous plasma components were checked during the validation. Selectivity was performed by testing six batches of di-potassium ethylene diamine tetraacetic acid blank plasma and extracted blank plasma gave good selectivity of Garenoxacin mesylate and internal standard. No interferences were found at the retention times of analyte and internal standard. Typical representative chromatograms of standard blank and blank with an internal standard sample using pooled plasma, from (Fig. 2 to 5).

**Linearity:** Calibration was found to be linear over the concentration range of 0.04 to 4 µg /ml for Garenoxacin mesylate. The coefficient correlation ($R^2$) value was found consistently greater than 0.99 this indicating linearity of results and an excellent correlation between peak area ratios for each concentration of Garenoxacin mesylate.

A representative calibration curve is shown in Fig. 6, which is obtained during the third precision and accuracy batch. Back-calculated concentrations obtained for 3 calibration curves are summarized in Table 2.

**Precision and Accuracy**

The intraday and interday accuracy and precision was assessed by analyzing six replicates at five different QC levels like Lower Limit of Quantitation (LLOQC), Low (LQC), MQC and High (HQC). Accuracy and Precision method performance was evaluated by six replicate analyses for Garenoxacin mesylate at four concentration levels, i.e., 0.04 µg/ml (LLOQC), 0.12 µg/ml (LQC), 1.5 µg/ml (MQC) and 3.2 µg/ml (HQC). The intra-day and inter-day accuracy of plasma samples were assessed, and excellent mean % accuracy was obtained with range varied from 98.07 % to 101.67 % for intraday and 97.30 % to 101.67 % for interday respectively. The
precision (%CV) of the analyte in plasma samples were calculated and found to be 1.82 % to 7.77 % for intraday and 3.49 % to 6.63 % for interday respectively. The results are summarized in Table 3 to 8.

Recovery: Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with a known amount of Garenoxacin mesylate. The recoveries obtained for Garenoxacin mesylate at LQC, MQC and HQC was found to be 99.72 %, 98.13 % respectively. The results are summarized in Table 8. The overall % mean recovery for % and 99.07 % respectively.

| Table 1: System Suitability of Garenoxacin mesylate |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S.NO | Analyte Area | Analyte RT (min) | IS Area | IS RT (min) | Area Ratio |
|-----|--------------|-----------------|----------|-------------|------------|
| 1  | 280210       | 3.41            | 755240   | 4.08        | 0.3710     |
| 2  | 280573       | 3.42            | 75487    | 4.07        | 0.3717     |
| 3  | 279867       | 3.42            | 755444   | 4.07        | 0.3705     |
| 4  | 280846       | 3.42            | 755112   | 4.08        | 0.3719     |
| 5  | 281245       | 3.41            | 755783   | 4.08        | 0.3721     |
| 6  | 280846       | 3.42            | 755854   | 4.07        | 0.3716     |
| Mean | NA          | 3.418            | NA       | 4.075       | 0.37146    |
| SD  | NA          | 0.0033           | NA       | 0.0047      | 0.000616   |
| %CV | NA          | 0.10             | NA       | 0.12        | 0.17       |

| Table 2: Linearity of Garenoxacin mesylate |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S.NO | STD1 | STD2 | STD3 | STD4 | STD5 | STD6 | STD7 | STD8 |
|-----|------|------|------|------|------|------|------|------|
| 1  | 0.044 | 0.078 | 0.114 | 0.992 | 1.499 | 1.942 | 3.282 | 3.956 |
| 2  | 0.038 | 0.082 | 0.126 | 1.014 | 1.534 | 2.098 | 2.994 | 4.128 |
| 3  | 0.040 | 0.081 | 0.122 | 0.989 | 1.519 | 2.114 | 3.095 | 4.056 |
| n  | 3     | 3     | 3     | 3     | 3     | 3     | 3     | 3     |
| Mean | 0.0407 | 0.0803 | 0.1207 | 0.9983 | 1.5173 | 2.0513 | 3.1237 | 4.0467 |
| SD  | 0.00306 | 0.00208 | 0.00611 | 0.01365 | 0.01756 | 0.09502 | 0.14612 | 0.08638 |
| %CV | 7.51 | 2.59 | 5.06 | 1.37 | 1.16 | 4.63 | 4.68 | 2.13 |
| %Mean | 101.67 | 100.42 | 100.56 | 99.83 | 101.16 | 102.57 | 97.61 | 101.17 |

| Table 3: Accuracy and Precision data for intra-day runs of Garenoxacin mesylate |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S.NO | HQC (3.2 µg/ml) | MQC (1.5 µg/ml) | LQC (0.12 µg/ml) | LLOQC (0.04 µg/ml) |
|-----|-----------------|---------------|----------------|------------------|
| 1  | 2.915 | 1.406 | 0.124 | 0.042 |
| 2  | 3.142 | 1.541 | 0.129 | 0.045 |
| 3  | 3.056 | 1.428 | 0.112 | 0.039 |
| 4  | 3.314 | 1.569 | 0.116 | 0.043 |
| 5  | 3.213 | 1.602 | 0.118 | 0.036 |
| 6  | 3.189 | 1.547 | 0.126 | 0.041 |
| n  | 6     | 6    | 6    | 6    |
| Mean | 3.1382 | 1.5155 | 0.1208 | 0.0410 |
| SD  | 0.13833 | 0.07954 | 0.00652 | 0.00318 |
| %CV | 4.41 | 5.25 | 5.40 | 7.77 |
| Mean Accuracy | 98.07 | 101.03 | 100.69 | 102.42 |

| Table 4: Accuracy and Precision data for intra-day runs of Garenoxacin mesylate |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| S.NO | HQC (3.2 µg/ml) | MQC (1.5 µg/ml) | LQC (0.12 µg/ml) | LLOQC (0.04 µg/ml) |
|-----|-----------------|---------------|----------------|------------------|
| 1  | 3.242 | 1.615 | 0.119 | 0.041 |
| 2  | 3.145 | 1.524 | 0.128 | 0.040 |
| 3  | 2.954 | 1.586 | 0.116 | 0.041 |
| 4  | 3.248 | 1.498 | 0.121 | 0.042 |
| 5  | 3.125 | 1.564 | 0.128 | 0.041 |
| 6  | 3.214 | 1.462 | 0.111 | 0.041 |
| n  | 6     | 6    | 6    | 6    |
| Mean | 3.1547 | 1.5415 | 0.1205 | 0.0408 |
| SD  | 0.11054 | 0.05728 | 0.00672 | 0.000074 |
| %CV | 3.50 | 3.72 | 5.57 | 1.82 |
| Mean Accuracy | 98.58 | 102.77 | 100.42 | 102.04 |
Ciprofloxacin Hydrochloride (Internal Standard) was found to be 97.86%.

Solution Stability

Long term stock solution stability

For Garenoxacin mesylate: In bench-top stability, six replicates of LQC & HQC samples (0.12 and 3.2 µg/ml) were analyzed after 9 hours at room temperature on the laboratory bench. The % means stability was calculated and found to 101.75 % for LQC and 98.04 % for HQC respectively. The long term stability of Garenoxacin is presented in the Table 9.

Matrix samples stability at -28±5 °C for 37 days & -80±5 °C

Long term stock solution stability for the Garenoxacin mesylate was determined at a concentration of LQC - HQC level after a storage period.
of 60 days at -28°C & -80°C in the refrigerator. The % mean stability of the Garenoxacin mesylate was found to be 97.42%, 98.77% & 100.10% and 99.34%.

The long term stability of Garenoxacin is presented in the Table. 10 & 11.

Table 8: Recovery- Garenoxacin mesylate

| S.NO | Unextracted Response | Extracted Response | Unextracted Response | Extracted Response | Unextracted Response | Extracted Response |
|------|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|
| 1    | 568986               | 565642            | 286421               | 280210            | 23011               | 22542             |
| 2    | 572584               | 565914            | 280717               | 280573            | 23510               | 23010             |
| 3    | 566425               | 567024            | 290246               | 279867            | 22653               | 22733             |
| 4    | 560123               | 566654            | 284124               | 280846            | 22716               | 22498             |
| 5    | 571681               | 568942            | 291415               | 281245            | 22834               | 23124             |
| 6    | 566540               | 562478            | 282670               | 280846            | 23101               | 22631             |
| n    | 6                    | 6                 | 6                    | 6                 | 6                    | 6                 |

Mean: 567723, 566109, 285932, 280598, 22971, 22756

SD: 4510.75, 2126.05, 4243.08, 495.41, 314.31, 256.30

% CV: 0.79, 0.38, 1.48, 0.18, 1.37, 1.13

Mean Recovery Overall: 98.72

% Mean Recovery Overall: 0.7948

SD: 0.80

Table 9: Long term stock solution stability Garenoxacin (after 9 hr)

| S. NO | HQC (3.2 µg/ml) | LQC (0.12 µg/ml) |
|-------|----------------|------------------|
| 1     | 2.992          | 0.112            |
| 2     | 3.019          | 0.113            |
| 3     | 3.214          | 0.122            |
| 4     | 3.162          | 0.127            |
| 5     | 3.219          | 0.131            |
| 6     | 3.218          | 0.128            |
| n     | 6              | 6                |

Mean: 3.1373, 0.1221

SD: 0.10468, 0.00797

% CV: 3.34, 6.52

% Mean accuracy: 98.04, 101.75

Table 10: Matrix samples stability at -28 ± 5°C temperature Garenoxacin (60 days)

| S.NO | HQC (3.2 µg/ml) | LQC (0.12 µg/ml) |
|------|----------------|------------------|
| 1    | 3.02           | 0.118            |
| 2    | 3.16           | 0.116            |
| 3    | 3.25           | 0.121            |
| 4    | 3.40           | 0.122            |
| 5    | 3.31           | 0.124            |
| 6    | 3.35           | 0.125            |
| n    | 6              | 6                |

Mean: 3.2473, 0.1210

SD: 0.14033, 0.00355

% CV: 4.32, 2.93

% Mean accuracy: 101.48, 100.93
CONCLUSION

The proposed RP-HPLC method for the estimation of Garenoxacin mesylate in human plasma is simple, precise, specific, highly accurate and less time consuming. So it can definitely be employed for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

REFERENCES

Aboli Edlabadkar, A. P. Rajput, Development of Four Simple UV-Spectrophotometry Methods for Estimation of Garenoxacin mesylate in Bulk Material and in Pharmaceutical Formulation. Der Pharmacia Lettre, 2016, 8, 213.

Aboli Edlabadkar, Ambarsing Rajput, A QbD Approach-RP-HPLC Method for Optimization, Development and Validation of Garenoxacin mesylate in Bulk and in Tablets., Eurasian Journal of Analytical Chemistry, 2018, 13(4), 26.

Agam Vora, Garenoxacin mesylate, Journal of the association of physicists of India, 2014, Vol 62.

Ashwinkumar B.Panchal, Bhumi R.Patel, Jaymin G.Patel, Yogesh K.Patel, Udayshankar J.Maurya, Development and validation of stability indicating RP-HPLC method for estimation of Garenoxacin mesylate in tablet dosage form, Int. J. of Pharm. Life Sci, April 2017, 5504-5510.

Gajjar, A. Bello, Z. Ge, L. Christopher, and D. M. Grasela Multiple-Dose Safety and Pharmacokinetics of Oral Garenoxacin mesylate in Healthy Subjects, 2003, DOI: 10.1128, Vol 47, No 7.

Hori S, Maki, Survey on the usage results of Garenoxacin mesylate tablets. Postmarketing surveillance report, 2011, 59.

ICH Q2B Validation of Analytical Procedures, Methodology International Conference on Harmonisation of Technical requirements for registration of Pharmaceuticals for Human use, Geneva, Switzerland, 1996.

Table 11: Matrix samples stability at -80±5°C- Garenoxacin (60 days)

| S.NO | Comparison samples | Stability samples | Comparison samples | Stability samples |
|------|-------------------|-----------------|-------------------|-----------------|
| 1    | 3.146             | 2.985           | 0.128             | 0.126           |
| 2    | 3.285             | 3.269           | 0.125             | 0.116           |
| 3    | 3.123             | 3.124           | 0.120             | 0.128           |
| 4    | 3.045             | 3.118           | 0.114             | 0.120           |
| 5    | 3.142             | 3.227           | 0.119             | 0.114           |
| 6    | 3.259             | 3.241           | 0.125             | 0.121           |
| n    | 6                 | 6               | 6                 | 6               |
| Mean | 3.1667            | 3.1607          | 0.1217            | 0.1209          |
| SD   | 0.08975           | 0.10641         | 0.00510           | 0.00543         |
| % CV | 2.83              | 3.37            | 4.19              | 4.49            |
| % Mean accuracy | 98.96 | 98.77 | 101.40 | 100.74 |