# Development And Validation of An Rp-Hplc Method For The Determination of Balofloxacin In Human Plasma

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**Abstract: A new simple, sensitive and stability indicating-HPLC method for the determination of Balofloxacin in pure and pharmaceutical dosage form was developed. Chromatographic separation was carried on Thermosil C18 (100*4.6 mm, 5 ), with a mobile phase composed of Methanol and pH 7 Triethylamine (70:30 V/V) at an absorption maxima 260 nm. Linearity for detector response was observed in the concentration range of 10-50 g/ml for Balofloxacin.. Correlation coefficient found to be 0.999. Retention time was found to be 3.706 min Balofloxacin.. Percent recovery studies were found in the range 99.0 – 101.0 % of test concentration. Drug product was exposed to acid, base, heat, oxidation and photolytic stress conditions and the samples were analysed by the proposed validated method. Results of the analysis were validated statistically and by recovery studies. The developed method was found to be precise for the determination of Balofloxacin in pure and its pharmaceutical dosage form.**

**Keywords: Balofloxacin, RP-HPLC, Stress degradation, Accuracy, Precision**

EXPERIMENTATION AND RESULTS

DRUG PROFILE - BALOFLOXACIN

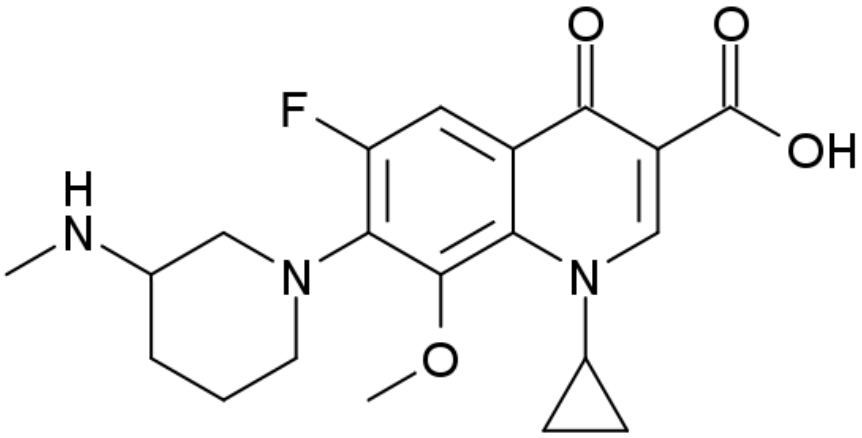



Fig.1 Structure of BALOFLOXACIN

Scheme: Rec.INN

CAS registry number (Chemical Abstracts Service): 0127294-70-6

Chemical Formula: $C_{20}H_{24}FN_3O_4$

Molecular Weight: 389

Therapeutic Category: Antibacterial: Fluoroquinolone (gyrase inhibitor)

Chemical Name: (±)-1-cyclopropyl-6-fluoro-1,4-dihydro-8-metoxy-7-[3-(metylamino)piperidino]-4-oxo-3-quinolinecarboxylic acid (WHO)

Foreign Names

- Balofloxacinum (Latin)
- Balofloxacin (German)
- Balofloxacine (French)
- Balofloxacino (Spanish)

EXPERIMENTATION AND RESULTS

A) MATERIALS

i) Instrumentation

The author has attempted to develop a liquid chromatographic method for the determination of BALOFLOXACIN in $K_2$EDTA human plasma using isocratic Shimadzu HPLC equipment comprising of binary LC 10AT

vp pumps, SIL 10AD vp Autosampler, CTO 10A vp column oven, an Phenomenex C18 column (150 X 4.6 mm id, 5 µm, ODS 2), and an SPD 10Avp UV-Visible detector. All the components of the chromatographic system were controlled using SCL-10A vp System controller. Data acquisition was done using LC Solutions ver. 1.23 SP 1 software. The details of the instruments used in the study are as follows:

| | |
|---|---|
| HPLC System | Shimadzu |
| Deep Freezer | Sanyo (-86°C) VIP Series |
| Microbalance | Sartorius |
| Vibramax | Heidolph |
| Vacuum pump | Millipore |
| Refrigerator | Samsung |
| pH meter | Orion |
| Micropipettes, Multipette and Micro tips | Brand and Eppendorf |
| Vortexer | Spinix |
| Refrigerated Centrifuge (-4$^{o}$C) | Heraeus |
| Poly propylene tubes | Tarson's |
| Water Purification System | Elix 10 / Milli-Q gradient |
| Ultra Sonicator | Power Sonic510, (Hwashin Technology) |
| Nitrogen Evaporator | Zymark Turbovap LV station, Caliper |

ii) Drug and Internal standard

The reference sample of BALOFLOXACIN (Purity 99.90 % w/w) was gifted by M/s Hetero Drugs Pvt Ltd., Hyderabad. CIPROFLOXACIN (Purity 99.80 % w/w) is gifted by M/s Roorkee Drugs Pvt Ltd.

iii) Chemicals and solvents

Acetonitrile (HPLC grade)
Methanol (HPLC grade)
Potassium Dihydrogen phosphate (AR Grade)
Orthophosphoric acid (GR grade)
Milli-Q water
Human $K_2$EDTA plasma
0.45µ Membrane filter

The linearity range of the drug was checked with the Q-Test at 95 percent confidence limits which was found to be well within the acceptable limits.

| Calibration Curve Standards table | |
|---|---|
| Points | Concentration (ng/ml) |
| STD 1 | 55.44 |
| STD 2 | 110.89 |
| STD 3 | 739.26 |
| STD 4 | 1478.52 |
| STD 5 | 2217.78 |
| STD 6 | 3326.67 |
| STD 7 | 4657.34 |
| STD 8 | 5082.41 |

| For Lowest Concentration | |
|---|---|
| Range | 5026.97 (STD 8- STD 1) |
| Module D1 | 55.45 (STD 1- STD 2) |
| Ratio Q1 | 0.01103 (D1/Range) |
| Q 95% | Theoretical value 0.52 |
| For highest Concentration | |
| Range | 5026.97 (STD 8- STD 1) |
| Module D2 | 425.07 (STD 8- STD 7) |
| Ratio Q2 | 0.08455 (D2/Range) |
| Q 95% | Theoretical value 0.52 |

iv) Stock solutions of the drug and the internal standard

*a) Drug stock solution of the drug*

About 100.0 mg of BALOFLOXACIN was weighed accurately and transferred into a 10 mL volumetric flask containing 5 mL of methanol. The contents were sonicated for 5 min and then the volume made up with a further quantity

of methanol to get an approximate concentration of 10.0 mg/mL. The stock is then stored in the refrigerator below $10^0$C until further use.

*b) Stock solution of the internal standard*

About 500.0 mg of CIPROFLOXACIN was weighed accurately and transferred into a 10 mL volumetric flask containing 5 mL of methanol. The solution was sonicated for 5 min and then the volume made up with a further quantity of the methanol to get an approximate concentration of 50.0 mg/mL. Store this stock solution below $10^0$C in a refrigerator.

*c) Internal standard dilution*

1.0 mL of CIPROFLOXACIN stock solution (1.0 mg/mL) was transferred into a 10 mL volumetric flask and the volume made up with a mixture of methanol and water (50:50 % v/v) to obtain a final concentration of 500 µg/mL. The solution was stored at room temperature and used within 8 hrs for analysis for spiking plasma samples.

v) Calibration Curve dilutions (CC Spiking solutions)

The calibration curve dilutions were prepared from BALOFLOXACIN stock solution as per the table given below in the concentration range of 1.11 to 101.65 µg/mL using a mixture of methanol and water (50:50) as the diluent. These dilutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.

*Spiked Calibration Curve Plasma Standards*

The above calibration curve dilutions (CC spiking solutions) were used to spike the screened blank human plasma matrix to prepare the plasma calibration curve standards ranging from 55.44 to 5082.41 ng/mL as per the table given below. Aliquots containing 0.500 mL of the above plasma calibration curve standards were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at –$70^0$C.

Preparation of Aqueous Calibration Curve Standards

| Name of the solution taken | Conc'in µg/mL | Volume taken in mL | Diluent Volume in mL | Total Volume in mL | Final Conc'n in µg/mL | Aqueous Std ID |
|---|---|---|---|---|---|---|
| BAL-ST | 9990.00 | 0.37 | 9.63 | 10.00 | 369.63 | BAL-INT |
| BAL-INT | 369.63 | 1.375 | 3.625 | 5.000 | 101.648 | AQ-CC-08 |
| BAL-INT | 369.63 | 1.260 | 3.740 | 5.000 | 93.147 | AQ-CC-07 |
| BAL-INT | 369.63 | 0.900 | 4.100 | 5.000 | 66.533 | AQ-CC-06 |
| BAL-INT | 369.63 | 0.600 | 4.400 | 5.000 | 44.356 | AQ-CC-05 |
| BAL-INT | 369.63 | 0.400 | 4.600 | 5.000 | 29.570 | AQ-CC-04 |
| BAL-INT | 369.63 | 0.200 | 4.800 | 5.000 | 14.785 | AQ-CC-03 |
| BAL-INT | 369.63 | 0.030 | 4.970 | 5.000 | 2.218 | AQ-CC-02 |
| BAL-INT | 369.63 | 0.015 | 4.985 | 5.000 | 1.109 | AQ-CC-01 |

Preparation of Plasma Spiked Calibration Standards

| Name of the solution taken | Conc'n in µg/mL | Volume taken in mL | Volume of Plasma taken in mL | Total Volume in mL | Final Concentration in ng/mL | Spiked Std ID |
|---|---|---|---|---|---|---|
| AQ-CC-08 | 101.648 | 0.500 | 9.500 | 10.000 | 5082.413 | CC-08 |
| AQ-CC-07 | 93.147 | 0.500 | 9.500 | 10.000 | 4657.338 | CC-07 |
| AQ-CC-06 | 66.533 | 0.500 | 9.500 | 10.000 | 3326.670 | CC-06 |
| AQ-CC-05 | 44.356 | 0.500 | 9.500 | 10.000 | 2217.780 | CC-05 |
| AQ-CC-04 | 29.570 | 0.500 | 9.500 | 10.000 | 1478.520 | CC-04 |
| AQ-CC-03 | 14.785 | 0.500 | 9.500 | 10.000 | 739.260 | CC-03 |
| AQ-CC-02 | 2.218 | 0.500 | 9.500 | 10.000 | 110.889 | CC-02 |
| AQ-CC-01 | 1.109 | 0.500 | 9.500 | 10.000 | 55.445 | CC-01 |

vi) Quality control dilution (QC Spiking solutions)

The quality control dilutions (QC spiking solutions) from BALOFLOXACIN stock solution were prepared as per the table given below in the concentration range from 1.18 to 81.32 µg/mL using a mixture of methanol and water (50:50) as the diluent. These dilutions (QC spiking solutions) were subsequently used for spiking the screened blank plasma.

*Spiked QC Plasma Samples*

The above quality control dilutions (QC spiking solutions) were used to spike the screened blank human plasma to prepare the plasma quality control plasma samples ranging from 59.14 to 4065.93 ng/mL as per the table given below. Aliquots containing 0.500 mL of the above plasma calibration curve standards were taken in pre labeled polypropylene vials which were then capped tightly and stored in a freezer at $-70^0$C.

Preparation of Aqueous Quality Control Samples

| Name of the solution taken | Conc'n in µg/mL | Volume taken in mL | Volume of Diluent taken in mL | Total Volume in mL | Final Conc'n in µg /mL | Aqueous QC ID |
|---|---|---|---|---|---|---|
| BOS-INT | 369.63 | 1.100 | 3.900 | 5.000 | 81.319 | AQS-HQC |
| BOS-INT | 369.63 | 0.7\10 | 4.290 | 5.000 | 52.487 | AQS-MQC |
| BOS-INT | 369.63 | 0.050 | 4.950 | 5.000 | 3.696 | AQS-LQC |
| BOS-INT | 369.63 | 0.016 | 4.984 | 5.000 | 1.183 | AQS-LLOQ QC |

Preparation of Plasma spiked Quality Control Samples

| Name of the solution taken | Conc'n in µg/mL | Volume taken in mL | Volume of Plasma taken in mL | Total Volume in mL | Final Conc'n in ng/mL | Spiked QC ID |
|---|---|---|---|---|---|---|
| AQS-HQC | 81.319 | 0.500 | 9.500 | 10.000 | 4065.930 | HQC |
| AQS-MQC | 52.487 | 0.500 | 9.500 | 10.000 | 2624.373 | MQC |
| AQS-LQC | 3.696 | 0.500 | 9.500 | 10.000 | 184.815 | LQC |
| AQS-LLOQ QC | 1.183 | 0.500 | 9.500 | 10.000 | 59.141 | LLOQ QC |

vii) Preparation of Solutions

20 mM Sodium acetate buffer (pH 3.0 ± 0.05)

About 1.36 grams of Potassium dihydrogen phosphate was weighed accurately and transferred into a 1000 mL reagent bottle and dissolved in 200mL of Milli-Q water. The above solution was sonicated for 5 min and its pH was adjusted to (3.0 ± 0.05) with orthophosphoric acid solution and made upto volume with Milli-Q water. The solution was stored at room temperature and used within 3 days from the date of preparation.

Mobile Phase

The composition of the mobile phase is a mixture of 40 parts of 10 mM Potassium dihydrogen orthophosphate buffer and 60 parts of methanol operated using a binary HPLC. The solution was stored at room temperature and used within 7 days from the date of preparation.

Diluent

500 mL of methanol was transferred into a 1000 mL reagent bottle and 500 mL of Milli-Q water was added to this, mixed and sonicated for 5 minutes. The solution was stored at room temperature and use within 7 days from the date of preparation.

Rinsing solution

500 mL of acetonitrile was transferred into a 1000 mL reagent bottle, 500 mL of Milli-Q water was added, mixed and sonicated for 5 minutes. The solution was stored at room temperature and used within 7 days from the date of preparation. This solution was used for rinsing the injection needle of the HPLC instrument.

For developing the method for the assay of BALOFLOXACIN, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A non-polar Phenomenex ODS-2, $C_{18}$ column (150 X 4.6 mm id) was chosen as the stationary phase for this study.

The mobile phase and the flow rate

In order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate.

To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a $C_{18}$

stationary phase. A binary mixture of 10 mM potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05) and methanol in a ratio of 40:60 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 1.0 mL/min was found to be suitable in the present study.

Detection wave length

The detection of the drug was monitored at 295 nm.

Retention time of drug & Internal standard

A model chromatogram showing the separation of BALOFLOXACIN is presented in Fig 4. Under the above optimized conditions retention times of 3.10 and 6.38 min were obtained for BALOFLOXACIN respectively.

Data acquisition and processing

The chromatograms were obtained and data were processed by the peak area ratio method using the LC solution software. The concentrations of the unknown samples were calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1\backslash X^2$ as weighting factor.

$$Y = m X + C$$

X = Analyte concentration / Internal standard concentration

Y = Analyte area / Internal standard area (area ratio)

m = Slope of the calibration curve

C = Y intercept value

Optimized Chromatographic Conditions

| Parameter | Specifications |
|---|---|
| Column | Phenomenex ODS-2, $C_{18,}$ (4.6 X 150 mm, 5µ) |
| Mobile phase | 10 mM Potassium dihydrogen phosphate buffer (pH 3.0) and Methanol (40: 60 v/v) |
| Flow rate | 1.0 mL/min |
| Run time | 7.5 min |
| Column oven temperature | Ambient |
| Auto sampler temperature | $4^0$C |
| Volume of injection | 20 µL |
| Detection wave length | 295 nm |

| | |
|---|---|
| Retention time of CIPROFLOXACIN | 3.10 min. |
| Retention time of BALOFLOXACIN | 6.38 min. |

Extraction process of plasma samples and their drying (Protein precipitation method)

Step 1: 300 micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred to a 2.0 ml eppendorf micro centrifuge tube.

Step: 2 To this 25 µL of CIPROFLOXACIN dilution (internal standard; approximately 500 µg/mL) was added and vortexed for ten seconds.

Step 3: 1.2 ml of HPLC grade methanol is then added to precipitate the plasma proteins.

Step 4: The samples are then centrifuged for 15 minutes in a refrigerated centrifuge and then subjected to flash-freezing using a mixture of dry-ice and acetone.

Step 5: The supernatant is then transferred into another labeled polypropylene tubes and evaporated to dryness under nitrogen at 40$^{o}$C.

Step 6: The dried residue is reconstituted with 0.3 ml of mobile phase, vortexed thoroughly and transferred into autosampler vials for analysis. An injection volume of 20 µL is taken during final analysis.

REPRESENTATIVE CHROMATOGRAMS

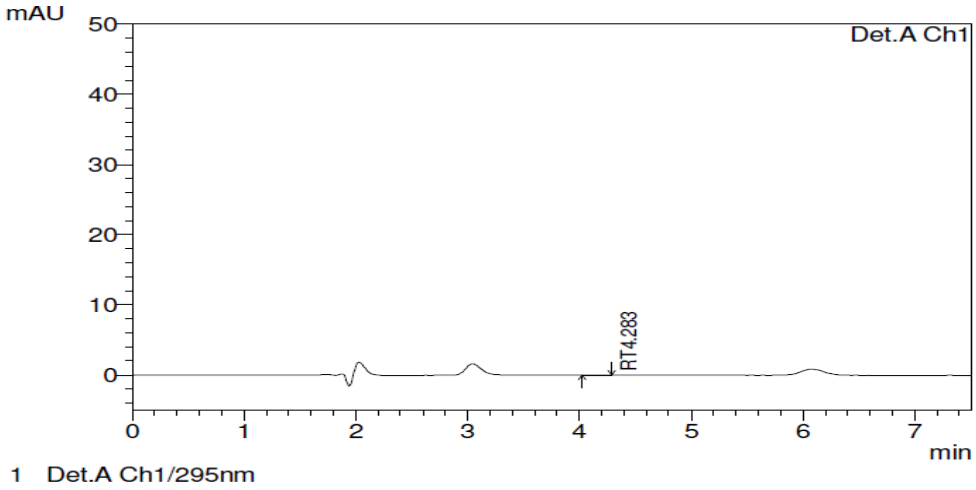



Fig. 2 A chromatogram of the extracted blank plasma sample

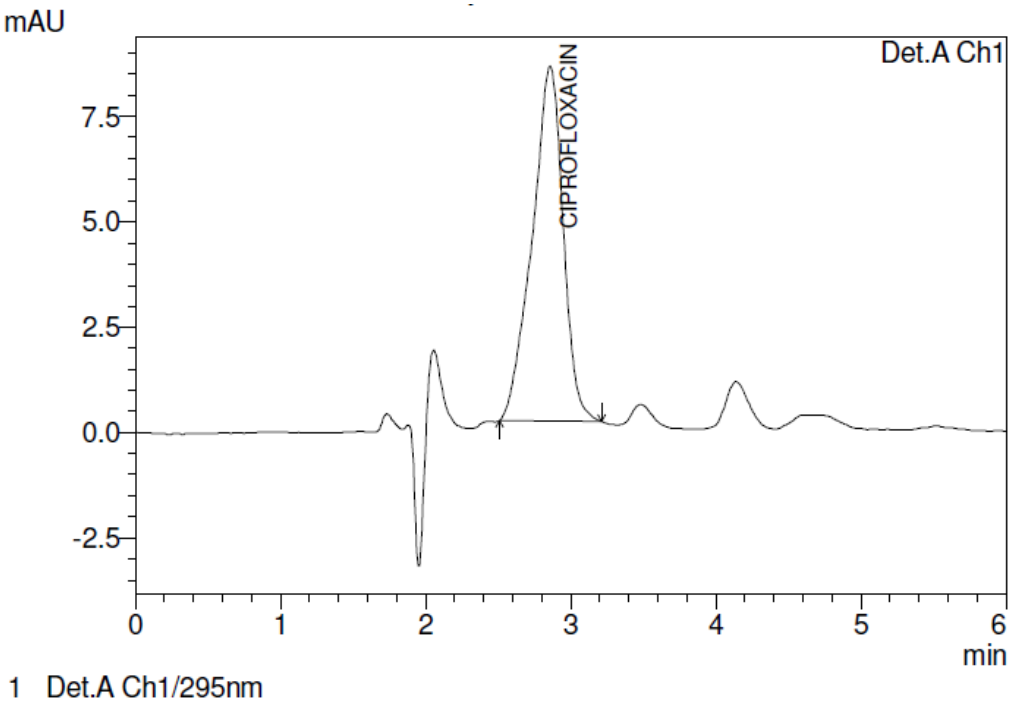
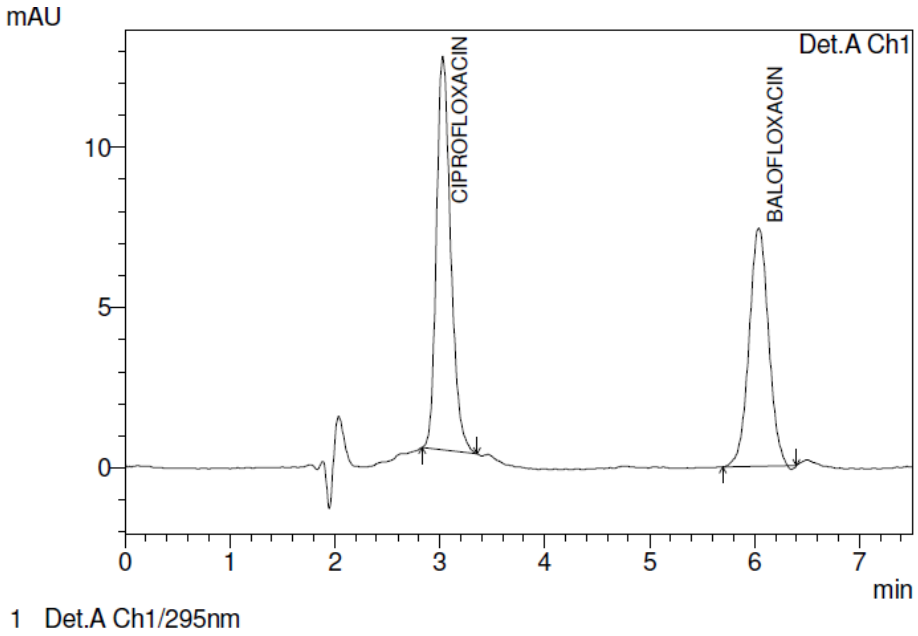



Fig. 3 Chromatogram of CIPROFLOXACIN (IS) spiked blank plasma sample




A chromatogram of BALOFLOXACIN (drug) and CIPROFLOXACIN (IS) extracted from human plasma

C) VALIDATION OF THE METHOD

i) Carry over test

This was determined by sequentially injecting the extracted blank plasma sample, the extracted ULOQ standard and once again the extracted blank plasma sample and calculating the percentage of the residual analyte and the internal standard carried over to the latter blank plasma sample.

Percent carryover of the drug and the internal standard

| Sample Name | Area at the RT of BALOFLOXACIN | % Carry Over of BALOFLOXACIN | Area at the RT of Ciprofloxacin (ISTD) | % Carry Over of ISTD |
|---|---|---|---|---|
| Mobile phase/Reconstitution Solution-I | 0 | 0.0 | 0 | 0.0 |
| Highest Aqueous standard(AQS ULOQ) | 2965488 | | 3126545 | |
| Extracted blank-I | 0 | 0.0 | 0 | 0.0 |
| Highest Extracted standard(Ext ULOQ) | 319886 | | 315644 | |

ii) Screening of plasma lots and specificity

The selectivity of the present method was evaluated by checking the blank EDTA (Ethylene di-amine tetra acetic acid) plasma (without spiking with BALOFLOXACIN) obtained from different blood donors. Six different lots of blank plasma were screened and all of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study.

Percent interferences at the retention times of the drug and the internal standards

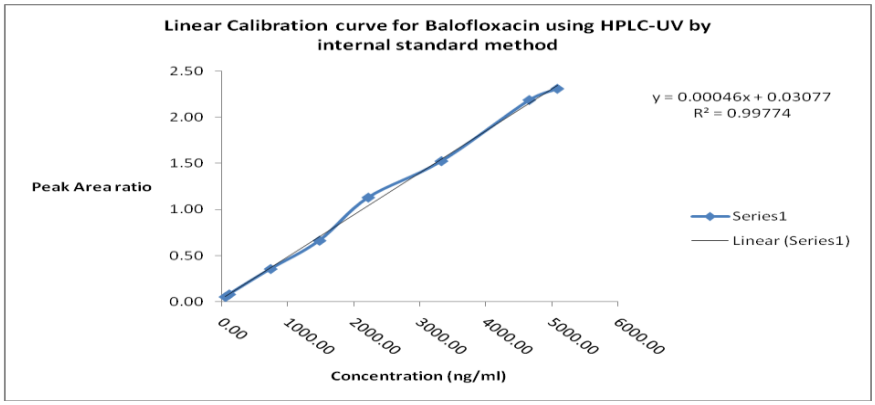
| SCREENING OF PLASMA LOTS AND SPECIFICITY | | | | | | |
|---|---|---|---|---|---|---|
| HUMAN PLASMA ID | BALOFLOXACIN | | | INTERNAL STANDARD | | |
| | RESPONSE IN BLANK | RESPONSE IN LLOQ | % INTERFERENCE | RESPONSE IN BLANK | RESPONSE IN LLOQ | % INTERFERENCE |
| 1 | 356 | 15654 | 2.27 | 655 | 299878 | 0.22 |
| 2 | 565 | 14988 | 3.77 | 767 | 301278 | 0.25 |
| 3 | 745 | 15897 | 4.69 | 1022 | 301654 | 0.34 |
| 4 | 612 | 16453 | 3.72 | 1019 | 312788 | 0.33 |
| 5 | 485 | 16676 | 2.91 | 898 | 309867 | 0.29 |
| 6 | 496 | 15934 | 3.11 | 965 | 299874 | 0.32 |
| Average | 543.2 | 15933.7 | 3.412 | 887.7 | 304223.2 | 0.292 |
| Total Number of Matrices | 6 | Number of matrices meeting the requirements | | | | 6 |
| Percentage of Matrices meeting the selectivity criteria | | | | | | 100 |

iii) Linearity

The linearity of the method was determined by a weighted ($1/X^2$ where X is concentration) least square regression analysis of the standard plots associated with the eight point standard curve for BALOFLOXACIN. The calibration line was linear in the range of 55.44 to 5082.41 ng/mL of the drug as shown in Fig 5. A straight-line fit made through the data

points by least square regression analysis showed a constant proportionality with minimal data scattering. The regression coefficient ($r^2$) ranged from 0.99774 to 0.99979 for BALOFLOXACIN.

Calibration curve for BALOFLOXACIN (P & A - 01)




iv) Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation for QC samples of BALOFLOXACIN. The accuracy of the assay was measured by computing the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentage nominal.

v) Recovery

*a) Recovery of the drug*

The percent recoveries were determined by comparing the areas of the extracted QC samples with equivalent aqueous samples. Mean Recovery for BALOFLOXACIN ranges from 7.137 % to 8.283% (Mean Recovery: 7.79%).

*b) Recovery of the internal standard*

The percent recovery of the IS (CIPROFLOXACIN) was determined by comparing the areas of the extracted IS samples with equivalent aqueous IS samples. The mean recovery obtained for CIPROFLOXACIN ranged from 8.619 % to 9.858 %. (Mean Recovery: 9.39 %)

Recovery of BALOFLOXACIN in human plasma

| RECOVERY OF DRUG IN HUMAN PLASMA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SR. NO | EXTRACTED HQC | AQS HQC | % RECOVERY | EXTRACTED MQC | AQS MQC | % RECOVERY | EXTRACTED LQC | AQS LQC | % RECOVERY |
| 1 | 203332 | 2564051 | 7.93 | 114766 | 1564901 | 7.33 | 13282 | 186180 | 7.13 |
| 2 | 212078 | 2430169 | 8.73 | 121067 | 1394127 | 8.68 | 11056 | 155368 | 7.12 |
| 3 | 220129 | 2692556 | 8.18 | 114685 | 1474022 | 7.78 | 10541 | 146879 | 7.18 |
| 4 | 202722 | 2549949 | 7.95 | 111036 | 1551130 | 7.16 | 13209 | 185621 | 7.12 |
| 5 | 211760 | 2422878 | 8.74 | 120002 | 1384508 | 8.67 | 11023 | 155135 | 7.11 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 219535 | 2685286 | 8.18 | 113894 | 1426116 | 7.99 | 10513 | 146482 | 7.18 |
| N | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| AVERAGE | 211592.6 | 2557481.6 | 8.283 | 115908.2 | 1465800.6 | 7.935 | 11603.9 | 162611.0 | 7.137 |
| SD | 7525.09 | 117478.07 | 0.36 | 3846.18 | 78089.79 | 0.65 | 1292.35 | 18444.28 | 0.03 |
| % CV | 3.56 | 4.59 | 4.40 | 3.32 | 5.33 | 8.15 | 11.14 | 11.34 | 0.44 |
| MEAN RECOVERY | 7.79 | | | | | | | | |
| SD | 0.587 | | | | | | | | |
| % CV | 7.54 | | | | | | | | |

Recovery of CIPROFLOXACIN (Internal standard)

| RECOVERY OF INTERNAL STANDARD IN HUMAN PLASMA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| SR. NO | EXTRACTED HQC | AQS HQC | % RECOVERY | EXTRACTED MQC | AQS MQC | % RECOVERY | EXTRACTED LQC | AQS LQC | % RECOVERY |
| 1 | 254551 | 2826725 | 9.01 | 262530 | 2785873 | 9.42 | 282420 | 3350899 | 8.43 |
| 2 | 279003 | 2707488 | 10.30 | 273788 | 2670078 | 10.25 | 293336 | 3421028 | 8.57 |
| 3 | 312708 | 2976778 | 10.50 | 264486 | 2802055 | 9.44 | 291456 | 3371526 | 8.64 |
| 4 | 253787 | 2811178 | 9.03 | 253998 | 2761357 | 9.20 | 280867 | 3340846 | 8.41 |
| 5 | 278584 | 2842862 | 9.80 | 271379 | 2651654 | 10.23 | 308003 | 3415896 | 9.02 |
| 6 | 311864 | 2968741 | 10.50 | 262661 | 2710988 | 9.69 | 290669 | 3362423 | 8.64 |
| N | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| AVERAGE | 281749.6 | 2855628.7 | 9.858 | 264806.9 | 2730334.3 | 9.706 | 291125.1 | 3377103.1 | 8.619 |
| SD | 26094.04 | 102363.48 | 0.70 | 7079.23 | 62267.88 | 0.44 | 9706.45 | 33710.96 | 0.22 |
| % CV | 9.26 | 3.58 | 7.11 | 2.67 | 2.28 | 4.58 | 3.33 | 1.00 | 2.56 |
| MEAN RECOVERY | 9.39 | | | | | | | | |
| SD | 0.676 | | | | | | | | |
| % CV | 7.19 | | | | | | | | |

vi) STABILITY

A) STABILITY OF THE DRUGS IN STOCK SOLUTION

*i) Short-term stability of the drugs in stock solution*

Stock solutions of about 1mg/mL of BALOFLOXACIN and CIPROFLOXACIN (IS) were prepared freshly in methanol and a portion of the freshly prepared stock solutions (Stability samples) were kept at a room temperature of ~25$^0$C for 9.00 hrs. The remaining portions of the above stock solutions were left in refrigerator below 10$^0$C, which were used as comparison samples.

The Short-term stability of the stock solution of BALOFLOXACIN and CIPROFLOXACIN in methanol was assessed by comparing the mean of the responses of six replicates of the stability samples with that of the six replicates of the comparison samples. After keeping for 9.00 hrs at a room temperature of ~ $25^0$C, the percent stabilities were found to be 103.05 % and 102.09 % for BALOFLOXACIN and CIPROFLOXACIN respectively.

*ii) Long-term stability of drugs in stock solution*

Stock solutions of about 1mg/mL of BALOFLOXACIN and CIPROFLOXACIN were prepared freshly in methanol and a portion of the stock solutions were stored in a refrigerator below $10^0$C (Stability samples) for 11 days. On the day of long term stability analysis, the stock solutions were freshly prepared and used as comparison samples.

The long-term stock solution stability of BALOFLOXACIN and CIPROFLOXACIN in methanol was assessed by comparing the mean of the responses of six replicates of the stability samples with that of the six replicates of the comparison samples. After keeping for 11 days in a refrigerator below $10^0$C, the percent stabilities obtained were 103.05 % and 102.96 % for BALOFLOXACIN and CIPROFLOXACIN respectively.

B) STABILITY OF DRUGS IN BIOLOGICAL MATRIX

Freeze-thaw stability

The Freeze-thaw stability of BALOFLOXACIN in human plasma was assessed by analyzing six replicates of the quality control samples at low and high concentration levels (Stability samples), previously frozen and thawed over 3 cycles along with six replicates of the freshly spiked (FS) quality control samples (Comparison samples) at low and high concentration levels (known from a freshly prepared calibration curve). The percent stability at low and high quality control concentration levels was calculated by comparing the mean of the concentrations of stability samples with that of the comparison samples.

The Freeze-thaw stability values for the calibration curve standards of BALOFLOXACIN in plasma after 3 FT cycles were 95.97 % and 96.98 % at low and high concentrations respectively.

Long-term stability in plasma matrix

For finding out the long-term stability of BALOFLOXACIN in human plasma the stability samples were stored at a temperature of -70ºC for 15 days. Then they were analyzed along with six replicates of the freshly prepared quality control samples (Comparison samples) at low and high concentration levels. The low and high concentration levels were read from the calibration curve. The quality control samples and the calibration curve standards were prepared by spiking the freshly prepared drug dilutions in screened blank plasma. The percent stabilities at low and high QC concentration levels were calculated by using the mean of the concentrations of the stability samples and the mean of the comparison samples.

The long-term stability values obtained for BALOFLOXACIN in human EDTA plasma at a temperature of -70°C for 20 days were 97.87 % and 99.64 % at high and low concentrations respectively.

Long-term stability of BALOFLOXACIN (20 days) at -70$^0$C in EDTA

| LONG-TERM STABILITY 20 DAYS AT -70 deg C | | | | |
|---|---|---|---|---|
| ALIQUOT ID | Obtained concentration ng/ml | | | |
| | FRESH HQC | STABILITY HQC | FRESH LQC | STABILITY LQC |
| 1 | 3985.42 | 3937.81 | 176.54 | 173.42 |
| 2 | 3999.61 | 4076.59 | 178.98 | 187.52 |
| 3 | 4096.55 | 3940.17 | 177.53 | 165.65 |
| 4 | 4056.56 | 4281.18 | 176.44 | 191.56 |
| 5 | 4044.11 | 3989.87 | 173.67 | 173.08 |
| 6 | 4016.87 | 4263.81 | 183.45 | 190.37 |
| N | 6 | 6 | 6 | 6 |
| AVERAGE | 4033.187 | 4081.572 | 177.768 | 180.266 |
| SD | 40.88 | 156.30 | 3.28 | 10.90 |
| % CV | 1.01 | 3.83 | 1.85 | 6.05 |
| NOMINAL CONC | 4059.83 | 4065.93 | 184.54 | 184.82 |
| ACCURACY | 100.66 | 99.62 | 103.81 | 102.53 |
| Correction Factor | 0.9985 | | 0.9985 | |
| % STABILITY | 101.05 | | 101.25 | |

vii) Dilution Integrity

A quality control pool (containing 6178.96 ng/mL of BALOFLOXACIN) was prepared in EDTA-plasma at a concentration of approximately twice the high CC standard (ULOQ) to assess the dilution integrity. The precision and accuracy for dilution integrity at 50% dilution and 25% dilution of the QC pool sample with screened blank human plasma were determined by using freshly spiked (FS) calibration curve standards. The precision for dilution integrity of BALOFLOXACIN was 0.85 % at 25 percent dilution and 1.98 % at 50 percent dilution. The accuracy for dilution integrity of BALOFLOXACIN was 98.59 % for 25 percent dilution and 99.07 % for 50 percent dilution. Results are shown in the following table.

Back calculated concentrations for quality control dilution samples in plasma

| DILUTION INTEGRITY | | |
|---|---|---|
| INJECTION NO | OBSERVED CONCENTRATION | |
| | 25 % Dilution | 50 % Dilution |
| 1 | 9877.54 | 9847.91 |
| 2 | 9945.68 | 9930.76 |
| 3 | 10105.68 | 10078.39 |
| 4 | 10117.88 | 10062.23 |
| 5 | 10245.66 | 10323.53 |
| 6 | 10566.78 | 10600.59 |
| N | 6 | 6 |
| AVERAGE | 10143.20333 | 10140.56932 |
| SD | 245.58 | 277.41 |
| % CV | 2.42 | 2.74 |
| NOMINAL CONC | 10164.82 | |
| ACCURACY | 99.79 | 99.76 |

4) SUMMARY OF THE RESULTS

| PARAMETERS | RESULTS |
|---|---|
| Screening of plasma lots and specificity | No significant interfering peaks were observed at the retention time of analyte (BALOFLOXACIN) and Internal standard (IS; CIPROFLOXACIN). |
| Calibration Curve | Calibration range: 55.44 to 5082.41ng/mL<br>Mean Accuracy (%nominal): 94.41 – 103.76 %<br>Precision (%CV): 0.94 – 6.08 %<br>Regression coefficient ($r^2$): 0.99774 - 0.99979 |
| Recovery | BALOFLOXACIN Mean % Recovery = 7.79 % ; % CV = 7.54<br>CIPROFLOXACIN (IS)<br>Mean % Recovery = 9.39 %, % CV = 7.19 |
| Precision (%CV) | Within-batch LLOQ QC: 4.01%<br>LQC, MQC & HQC: 4.66 – 5.09 %<br>Global LLOQ QC: 0.39 %<br>LQC, MQC & HQC: 0.36 – 0.42 % |
| Accuracy (% Nominal Conc.) | Within-batch LLOQ QC: 95.94 %<br>LQC, MQC & HQC: 100.24 – 103.06 %<br>Global LLOQ QC: 96.06 %<br>LQC, MQC & HQC: 100.31 – 103.13 % |
| Short term stock solution stability (9.0 hrs) | BALOFLOXACIN Percent stability: 103.05 %<br>CIPROFLOXACIN (IS) Percent stability: 102.09 % |
| Long term stock solution stability (11 Days) | BALOFLOXACIN Percent stability: 103.05 %<br>CIPROFLOXACIN (IS) Percent stability: 102.96 % |
| Freeze – Thaw stability (3Cycles) | Percent stability: At LQC Level: 99.97 %<br>At HQC Level: 99.82 % |
| Bench top stability (8.00 hrs) | Percent stability: At LQC Level: 100.58 %<br>At HQC Level: 100.39 % |
| In injector stability (48.00 hrs) | Percent stability: At LQC Level: 101.21 %<br>At HQC Level: 100.99 % |
| Long term stability in plasma (25 days at-70$^{o}$C) | Percent stability: At LQC Level: 101.25 %<br>At HQC Level: 101.05 % |
| Dilution Integrity | At 25% dilution: Precision: 2.42 % Accuracy: 99.79 %<br>At 50 % dilution: Precision: 2.74 % Accuracy: 99.76 % |

CONCLUSIONS

Based on the results obtained in this study, it is concluded that the present validated method can be successfully applied for the estimation of BALOFLOXACIN in human plasma over the concentration range of 55.44 to 5082.41 ng/mL. The method for determination of BALOFLOXACIN in human plasma using HPLC with UV detection met the acceptance criteria with respect to selectivity, precision, accuracy, linearity, recovery and dilution integrity.

Stability evaluations performed in EDTA human plasma, stock solutions and stock dilutions met the acceptance criteria, demonstrating insignificant degradation of BALOFLOXACIN over the specified storage durations and conditions.

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