How to Cite:
Deepti, A., & Sahithi, A. (2022). A sensitive bioanalytical method development and validation of afatinib in human plasma by LC-ESI-MS/MS. *International Journal of Health Sciences*, *6*(S5), 1736–1384. https://doi.org/10.53730/ijhs.v6nS5.9522

A sensitive bioanalytical method development and validation of afatinib in human plasma by LC-ESI-MS/MS

Asha Deepti
Assistant Professor, Department of Pharmaceutical Analysis GITAM Institute of Pharmacy, GITAM (Deemed to be) University, Visakhapatnam, Andhra Pradesh, India-530045

Alapati Sahithi
Research scholar, GITAM Institute of Pharmacy, GITAM (Deemed to be) University, Visakhapatnam, Andhra Pradesh, India-530045 & Assistant professor, Department of Pharmaceutical Analysis, School of Pharmacy, Nalla Narasimha Reddy Education Society's Group of Institutions, Hyderabad, Telangana, INDIA-500088.
Corresponding Author email: salapati1@gitam.in

***Abstract*---**A simple, sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of Afatinib in human plasma using Cabozantinib as an internal standard (IS). Chromatographic separation was performed on Water X Bridge c18 2.1x100 column with an isocratic mobile phase composed of acetonitrile and 0.2% Ammonia in water in the ratio of (70:30 v/v), at a flow rate of 0.250mL/min. Afatinib and Cabozantinib were detected with parent ions at m/z 486.36 to 370.90 and the daughter mass was found to be 381.45 to 304.93 in multiple reaction monitoring (MRM) positive mode respectively. The protein precipitation method was used to extract the drug and IS. The method was validated over a linear concentration range of 2.0-1000.0 ng/mL with a correlation coefficient (r2) ≥ 0. 9994. This method demonstrated Intra and inter-day Precision within 0.3 to 2.5 and 0.4 to 3.9 % and Accuracy from 96.55 to 105.45 and 95.26 to 110.6%. Afatinib was found to be stable throughout Long-term stability studies, benchtop, and postoperative stability studies.

***Keywords*---**Afatinib, Cabozantinib, Internal standard, Flow rate.

Introduction

Afatinib dimaleate1-4 is chemically known as (Z)-but-2- enedioic acid;(E)-N-[4-(3-chloro-4-fluoroanilino)-7-[(3S)- oxolan-3-yl]oxyquinazolin-6-yl]-4-(dimethylamino) but-2- enamide. Its molecular formula is $C_{32}H_{33}C_lFN_5O_{11}$ and its molecular weight is 718.083g/mol (Kawada I et., al 2008, Schiller JH et., al 2002, John T et ., al 2009, Olivier Bouche et., al 2011, Jahnavi Bandla et., al 2018).It is white to brownish-yellow powder and soluble in water. It has pKa values of 8.81 and 12.49. It is an anticancer drug used for the treatment of patients with metastatic non-small lung cancer(Madhavi S et., al 2018, Kishor Kumar Mule L et., al 2017). It acts as an irreversible covalent inhibitor of receptor tyrosine kinases epidermal growth receptor (EGFR) and erbB-2 (HER2) (Ravikumar Vejendla et., al 2015, Ngwa, G..et., al 2010).

Materials and Methods

Materials and Reagents

Afatinib and Cabozantinib (Internal Standard) were procured from Fisher chemicals, Mumbai, India. Acetonitrile of HPLC grade was procured from Rankem Ltd., India. The water of HPLC grade was obtained from Merck Specialties Private Limited, Mumbai, India. Ammonium formate and formic acid of HPLC grade were procured from Merck Specialties Private Limited, Mumbai, India.

Instrumentation

An LC-MS/MS method was performed on a liquid chromatographic system consisting of a Waters Acquity UPLC system coupled with a Water Quattro Premier XE mass spectrometer with electrospray ionization (ESI) used for analysis and Mass Lynx 4.1 SCN 805 software for processing and data collecting. Agilent, Zorbx, and XDB C18 (2.1 x 50 mm ID, 5 µm) are used as a stationary phase.

Standard solutions

Primary stock solutions of Afatinib for preparation of standard calibration curve and quality control (QC) samples were prepared from separate weighing. The stock solution of Afatinib (10 ng/mL) was prepared in acetonitrile and these stocks were stored at 2-8 °C. From these stock solutions, appropriate dilutions were made using acetonitrile, to produce working standard solutions of Afatinib.
A working concentration of the internal standard (10 ng/mL) solution was prepared in acetonitrile and refrigerated.

Preparation of calibration curve standards and quality control samples

A calibration curve consisting of a set of seven non-zero concentrations ranging from 2.0 to 1000 ng/mL of Afatinib was prepared. The QC samples were prepared for Afatinib 2.200 (LLOQ),4.400 (LQC),450 (MQC), and 750ng/mL (HQC). All the samples were incubated at 37 °C for subsequent use.

Sample processing

To 0.4ml of plasma, add 20ml of 10mg/ml internal standard (Cabozantinib) and vortex for 30sec, add 4ml of Methanol and vortex for 3 min, by using a multi-pulse vortexer. Then 2ml of the supernatant clear organic layer is transferred into a 7.5ml test tube and evaporated to dryness using Speed vapor at 40^0C under a stream of nitrogen. Then the dried extract is reconstituted with 200 ml of diluent (Methanol: Water 1:1) and a 20ml aliquot is injected into the chromatographic system.

Method validation

The method was validated as per FDA guidelines. The fundamental parameters for this validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability

Selectivity
For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, other matrices) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should been sure that the lower limit of quantification(LLOQ)

Accuracy, Precision Recovery
Accuracy of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of them from the true value serves as the measure of accuracy.
Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, Intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Recovery of the analyte need not be 100%, but the extent to recovery of an analyte and internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with un-extracted standards that represent 100% recovery.

Calibration Curve

A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known

concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen based on the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

Stability

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

Long-term stability
The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of blank-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.
Stock solution stability

The stability of stock solutions of drugs and the internal standard should be evaluated at room temperature for at least 6 h. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Benchtop Stability
It will be performed to evaluate the stability of the samples, which were kept on the bench during the extraction process. The anticipated time for the benchtop stability usually 4 to 24 hours should cover the duration of the time

Table 1. Optimized method development parameters of Afatinib

HPLC	Water Acquity UPLC
MASS	Water Quattro premier XE
ION SOURCE	Electrospray Ionization
COLUMN	Water X Bridge c18 2.1x100
COLUMN OVEN TEMPERATURE	30^0c
MOBILE PHASE	Acetonitrile: 0.2% Ammonia in water (70:30)
FLOW RATE	0.250 mL/min
VOLUME OF INJECTION	10µL

RETENTION TIME	Afatinib : 1.53 Cabozantinib :1.15

Table 2. Calibration curve samples of Afatinib in human plasma

CC ID	2ng/mL	4ng/mL	10ng/mL	50ng/mL	100ng/ml	500ng/mL	800ng/mL	1000ng/mL
1	188	354	621	2405	4681	23089	33369	37408
2	188	350	620	2404	4679	23088	33365	37405
3	187	355	622	2401	4682	23089	33366	37408
4	188	350	618	2405	4684	23087	33368	37407
5	186	353	621	2400	4681	23088	33369	37406
Mean	187.4	352.4	620.4	2403	4681.4	23088.2	33367.4	37406.8
SD	0.8	2.059	1.356	2.097	1.648	0.7483	1.6248	1.166
%CV	0.43	0.58	0.225	0.09	0.03	0.02	0.04	0.02

Results and Discussion

Mass spectrometry

Mass parameters were tuned in positive ionization modes for the analytes. Good response was achieved in positive ionization mode. Data from the MRM mode were considered to obtain better selectivity. Deprotonated form analyte and IS, [MeH] e ion have the m/z value of parent ion 486.36 to 370.90 respectively and the daughter mass was found to be 381.45 and 304.93 for the analyte and IS respectively.

Method development

A series of trials were conducted using Acetonitrile and ammonia having different pH to obtain the required separations. After reviewing the results, ammonia was selected as the buffer, and acetonitrile was employed as an organic solvent. Different ratios of the buffer and acetonitrile were tried and finally the acetonitrile: 0.2% Ammonia in water at70:30ratio was selected as an optimized mobile phase as it eluted a peak with good characteristics for both Afatinib as well as Cabozantinib (internal standard). (Figures 1&2). The developed method gave an asymmetric peak at a retention time of 1.53 min for Afatinib and 1.15 min for Cabozantinib and satisfied all the peak properties as per USP guidelines.

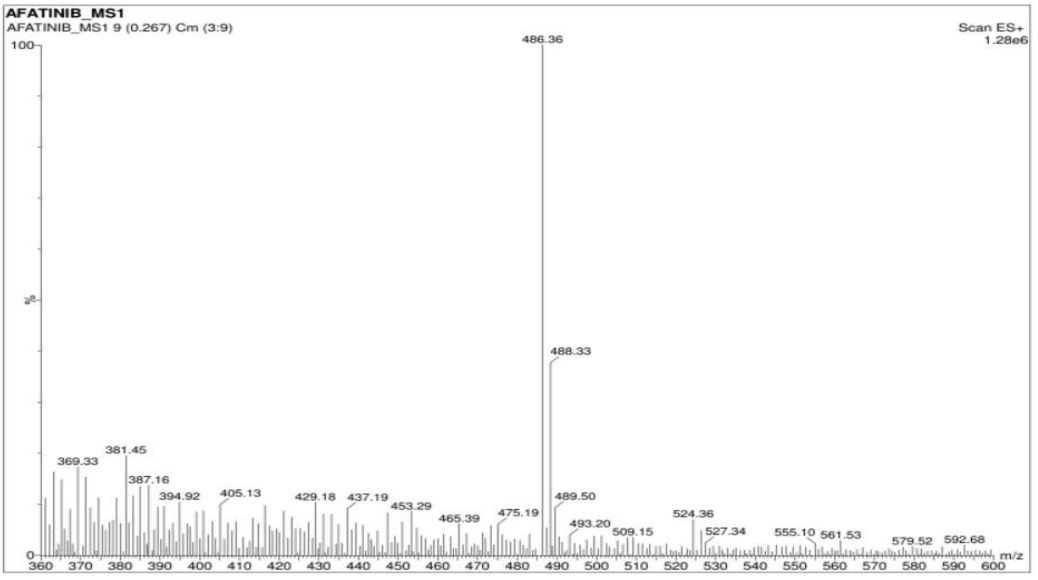

Figure 1: Mass spectra of Parent ion of Afatinib

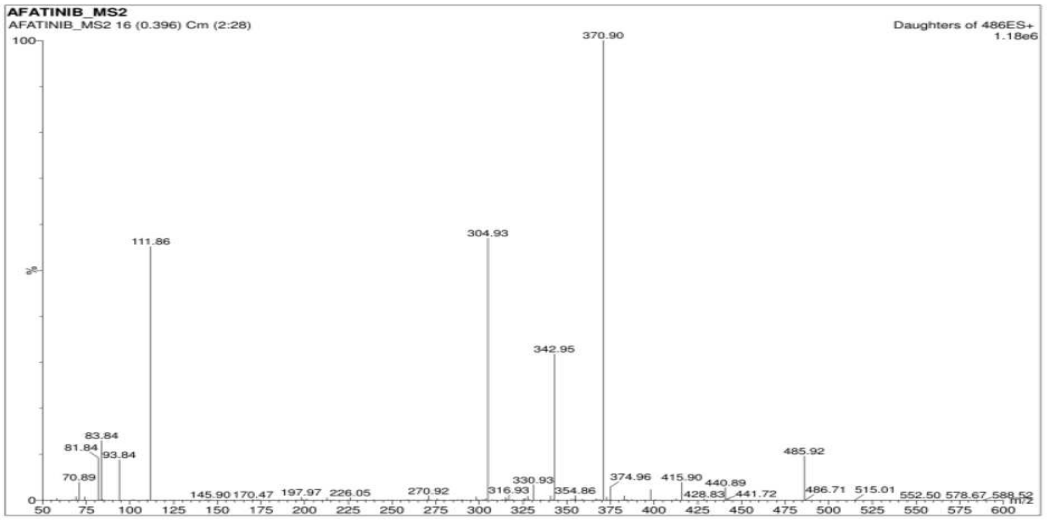

Figure 1: Mass spectra of Daughter ion of Afatinib

Linearity

To establish the range of Afatinib concentrations that can be assayed by using the present method, eight different sets containing Afatinib concentrations from 2.0 to 1000 ng/ml were taken and analyzed. The area ratio obtained against each concentration is plotted against the amount of Afatinib. A straight-line fit is made through the data points by the least square regression analysis and a constant proportionality is observed with minimal data scattering. The r2 value was consistently greater than 0.999 in all five cases. So, the Pazopanib can be successfully analyzed with the present system within this concentration.

Sensitivity

The lower limit of quantification is found to be 2 ng/ml for Afatinib. The between run accuracy and precision for Afatinib at 2 ng/ml were 99.5% respectively.

Precision

Precision was checked by three concentrations of LQC, MQC, and HQC (4.400, 450,750 ng/ml) against a single linearity curve, and the assay values to the actual value expressed in percentage. It can be observed from these tables that the intra-day or with run accuracy ranged from 92.23 to 102.8%. The between run or inter-day or total accuracy ranged between 99.15 and 101.4%.

Recovery

The percentage recoveries were determined by measuring the peak area of prepared plasma validation samples at a concentration of 4.400, 450, and 750 ng/ml respectively. The peak areas of the validation samples were compared to the peak area of the extracted blank plasma spiked with standards containing the same area concentrations of Afatinib. Recovery for Afatinib ranged between 98.33 and 103.58%.

Stability

Benchtop stability is measured since the plasma is taken out from the freezer and thawed. This study is necessary to avoid repeated access to deep freezers within shorter intervals. The thawed plasma samples are triplicated at each QC concentration are processed at 3-6 h and Afatinib concentration is measured. From tables, 2&3 it can be seen that Afatinib is highly stable in the present matrix. Since nearly 100.21, 101.25, and 100.05% concentrations are observed even after 6 hrs. In addition, the long-term stability of Afatinibin QC samples after 105 days of storage at -30oC was also evaluated. The concentrations ranged from 94.6 to 101.8% of the theoretical values. These results confirmed the stability of Afatinib in human plasma for at least 105 days at -30^{o}C.

Table: 3 Bench Top stability of Afatinib

S.NO	Concentration ng/mL					
	LQC [4.00ng/mL		MQC 450ng/mL		HQC 750ng/mL	
	Comparison	Stability	Comparison	stability	Comparison	stability
1	4.200	4.451	450.12	490.21	750.21	751.24
2	4.245	4.477	450.24	490.32	750.25	751.32
3	4.235	4.492	450.22	490.25	750.34	752.34
4	4.280	4.325	450.44	490.35	750.32	751.42
5	4.230	4.521	450.25	491.35	750.24	750.55
6	4.245	4.411	450.61	490.24	750.33	750.64
SD	0.0289	0.0287	0.1786	0.4424	0.0549	0.6456
% CV	1.26	2.48	0.04	0.07	0.01	0.04
% Difference	1.22		0.03		0.03	

Table 4. Long term stability of Afatinib

S.NO	Concentration ng/mL					
	LQC [4.00ng/mL		MQC 450ng/mL		HQC 750ng/mL	
	Comparison	Stability	Comparison	stability	Comparison	stability
1	4.200	4.500	450.12	450.21	750.21	751.24
2	4.245	4.600	450.24	451.32	750.25	750.32
3	4.235	4.492	450.22	450.25	750.34	751.34
4	4.280	4.258	450.44	451.35	750.32	751.42
5	4.230	4.580	450.25	451.35	750.24	750.75
6	4.245	4.414	450.61	452.24	750.33	750.80
SD	0.0289	0.1143	0.1786	0.7054	0.0549	0.6456
% CV	1.29	3.62	0.04	0.06	0.01	0.07
% Difference	2.33		0.02		0.06	

Conclusion

Based on the data presented in this report, it can be included that the present method is validated for the estimation of Afatinib in human plasma over a concentration range of 2.0-1000 ng/ml. The precision and accuracy are very much within the prescribed limits in this concentration range. Expected recoveries are observed in the present processing technique for LQC, MQC, and HQC.

Reference

1. Kawada I, Soejima K, Watanabe H, Nakachi I, Yasuda H, Naoki K, et al. An alternative method for screening EGFR mutation using RFLP in non-small cell lung cancer patients. *Journal of Thoracic Oncology* 2008;3:1096-1103.
2. Schiller JH. Small cell lung cancer: Defining a role for emerging platinum drugs. *Oncology* 2002;63(2):105-114.
3. John T, Liu G, Tsao MS. Overview of molecular testing in non-small cell lung cancer: mutational analysis, gene copy number, protein expression and other biomarkers of EGFR for the prediction of response to tyrosine kinase inhibitors. *Oncogene* 2009;28 Suppl 1:S14-23.
4. Olivier Bouche, Frederique Maindrault-Goebel, Michel Ducreux, Gerard Lledo, Thierry Andre, Peter Stopfer, et al. Phase II trial of weekly alternating sequential BIBF 1120 and afatinib for advanced colorectal cancer. *Anticancer Research*2011;31:2271-2282.
5. Jahnavi Bandla, Ganapaty S. New stability-indicating ultraperformance liquid chromatography method development and validation of lenvatinib mesylate in bulk drug and pharmaceutical dosage forms. *Asian Journal of Pharmaceutical and Clinical Research* 2018;11(9):140-143.
6. Madhavi S, Prameela Rani A. Simultaneous reverse phase ultra-performance liquid chromatography method development and validation for estimation of Grazoprevir and Elbasvir. *Asian Journal of Pharmaceutical and Clinical Research* 2018;11(4):100–104.

7. Kishor kumar Mule L. Rapid analytical method for assay determination for prochlorperazine edisylate drug substances by Ultra Performance Liquid Chromatography. *International Journal of Current Pharmaceutical Research* 2017;9(4):118- 122.
8. Ravikumar Vejendla, Subramanyam CVS, Veerabhadram G. New RP-HPLC method for the determination of Afatinib dimaleate in bulk and pharmaceutical dosage forms. *Indo American Journal of Pharmaceutical Research* 2015;5(5):2098-2111.
9. ICH, Q2B. Harmonized Tripartite Guideline, Validation of Analytical Procedure: Methodology, IFPMA, in: *Proceedings of the International Conference on Harmonization, Geneva*; 1996.
10. Ngwa, G.. (2010). Forced degradation as an integral part of HPLC stability-indicating method development. *Drug Delivery Technology*. 10. 56-59.