RESEARCH ARTICLE

PICOGRAM LEVEL QUANTIFICATION OF AFATINIB IN HUMAN PLASMA SAMPLES BY LC–ESI-MS/MS

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ARTICLE INFO	ABSTRACT
	A simple, sensitive and accurate liquid chromatography tandem mass spectrometric (LC/MS/MS) method has been developed and validated for determination of Afatinib in human plasma. Zorbax SB-C18, 4.6 x 75 mm, 3.5 μm, 80 Å column, 5mM ammonium formate: acetonitrile (10:90 v/v) mobile phase was used for Chromatographic separation of Afatinib. The Afatinib and Afatinib-D4 were monitored by electrospray ionization in positive ion multiple reaction monitoring (MRM) mode was used to detect the Afatinib at m/z 486.10 /112.10 and Afatinib-D4 (IS) at 490.90 /112.10. Liquid-liquid extraction was employed in the extraction of analytes from human plasma. This method is validated over a linear concentration range of 50.0–10000.0 pg/mL with a correlation coefficient (r) of ≥ 0.9997. Both drug and Internal standards (IS) were stable in plasma samples.

INTRODUCTION

Afatinib (AT) is presented as the dimaleate salt, with the chemical name N-(4-((3-Chloro-4-fluorophenyl)amino)-7-(((3S)tetrahydro-3-furanyl)oxy)-6-quinazolinyl)-4(dimethy lamino)-2-butenamide. Afatinib dimaleate is a white to brownish yellow powder, water soluble and hygroscopic, with an molecular formula of $C_{24}H_{25}ClFN_5O_3$ and a molecular weight of 485.93. It was used in the treatment for non small cell lung cancer (Ravikumar *et al.*, 2015). Literature review reveals that very few analytical methods have been reported for the determination of Afatinib which includes Pharmacokinetics studies (Miller *et al.*, 2012; Yang *et al.*, 2012; Sequist *et al.*, 2013; Wu *et al.*, 2014; Seiwert *et al.*, 2014; Stopfer *et al.*, 2012), High performance liquid chromatography (Ravikumar *et al.*, 2015), ultra-performance liquid chromatography-DAD (Helvenstein *et al.*, 2014; Marwa Fouad *et al.*, 2015), Liquid chromatography-Mass spectroscopy (Adnan A. Kadi *et al.*, 2016; Peter Stopfer *et al.*, 2012; Hideki Hayashi *et al.*, 2016). It is important to develop the superior bio analytical method with proper deuterated or analogue based internal standards interms of matrix effect and reproducibility. Moreover, Should not consider the runtime always to minimize the analysis rather than reproducibility and stability for long analytical batches. The present study describes, for the first time, the development and validation of an isocratic LC-MS/MS with highly efficient,

more specific and highly sensitive, simple extraction, good linear method for quantitative determination of AT in human plasma with the small amount of plasma usage.

MATERIALS AND METHODS

Chemicals and reagents

Afatinib (AT) reference standard (purity, 99.5%) was purchased from Symed labs, Afatinib -D4 was obtained from Torront research chemicals Canada (Fig.1). Tertiary butyl methyl ether (TBME), HPLC grade methanol and acetonitrile were purchased from J.T. Baker USA. Potassium dihydrogen phosphate (KH_2PO_4, reagent grade), Ammonium formate (reagent grade) was purchased from Merck Limited, Worli, Mumbai. Human plasma was obtained from Navajeevan blood bank, Hyderabad, India. Ultra pure water from MilliQ-system (Millipore) was used through the study.

Instrumentation

The 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany). Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).

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Detection

The mass transitions were selected as *m/z* 486.10/112.0 and *m/z* 490.90/112.10 for quantification of AT and ATD4 respectively (Figure-3 &4).

Chromatographic conditions

chromatographic separation was achieved with 5 mM ammonium formate: acetonitrile (10:90v/v), gave the best peak shape and low baseline noise was observed using the Zorbax SB-C18, 4.6 x 75 mm, 3.5 μm 80 Å. The total analysis time was 12 min and flow rate was set to 0.6 mL/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 20 μL for better ionization and chromatography. Afatinib-D4 (ATD4) was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. The analyte and internal standard were eluted at 9.2 (AT), 8.2 (ATD4) minutes with total runtime of 13 minutes for each injection.

Calibration standards and quality control Samples

Standard Stock solutions of AT (100.0 μg/mL) were prepared in methanol. From each stock solution 500.0 ng/mL, 25.0 ng/mL, 2.5 ng/mL intermediate dilutions were prepared in plasma. Aliquots of 500.0 ng/mL, 25.0 ng/mL and 2.5 ng/mL were used to spike blank human plasma in order to obtain calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0 and 10000.0 pg/mL. Four levels of QC concentrations at 50.0, 150.0, 3000.0 and 8000.0 pg/mL (LLOQ, LQC, MQC and HQC) were prepared by using the different plasma. Spiked calibration curve standards and Quality control standards were stored at -30 °C. Standard stock solutions of ATD4 (100.0 μg/mL) were prepared in methanol. ATD4 was further diluted to 30.0 ng/mL (Spiked concentration of internal standard) using 50% methanol and stored in the refrigerator 2-8 ^{0}C until analysis.

Sample preparation

Liquid-liquid extraction was carried out to extract the drug and IS for this purpose 100 μL of respective concentration of plasma sample was taken into polypropylene tubes and mixed with 50μL of internal standard (30.0 ng/mL). This was followed by addition of 100 μL of 10mM KH_2PO4 solution and 2.5 mL of methyl tertiary butyl ether and vortexed for approximately 5 minutes. Then the Samples were centrifuged at 4000 rpm for 10 minutes at 20°C. Further, the supernatant was transferred into labeled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with the reconstitution solution (Acetonitrile: 5mM ammonium formate (90:10)) and vortexed for 2 minutes. Finally, Sample was transferred into auto sampler vials to inject into the LC-MS/MS.

Selectivity and specificity

Selectivity was performed by analyzed the human blank plasma samples from six different sources (donors) with an additional hemolysed group and lipedimic group to test for interference at the retention times of analytes. The peak area of AT in blank samples should not be more than 20% of mean peak area of LOQ of AT. Similarly, peak area of ATd4 in a blank sample should not be more than 5% of mean peak area of LOQ of ATd4.

Precision and accuracy

Precision and accuracy was determined by replicate analysis of quality control samples (*n* = 6) at LQC (low quality control), MQC (medium quality control) and HQC (high quality control) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect

The matrix effect due to plasma was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pretreatment (Liquid-liquid extraction with MTBE) with that of the reconstituted samples. Experiments were performed at MQC levels in triplicate with six different plasma lots. The acceptable precision (%CV) of ≤ 15% was maintained.

Recovery

The extraction efficiencies of AT and ATd4 were determined by analysis of six replicates at each quality control concentration level and at one concentration for the internal standard ATd4. The percent recovery was evaluated by comparing the peak areas of extracted standards to the peak areas of non extracted standards (spiked into mobile phase).

Limit of detection and quantification (LOD and LOQ)

The limit of detection (LOD) is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. LOD was determined using the signal-to-noise ratio (s/n) of 3:1 by comparing test results from samples with known concentrations of analytes with blank samples. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOQ was found by analyzing a set of mobile phase and plasma standards with a known concentration of AT.

Stability (Freeze- thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed by comparing the area response of analyte and internal standard in the stability sample, with the area response of sample prepared from fresh stock solution. Stability studies in plasma were performed at the LQC and HQC concentration level using six replicates at each level. Analyte was considered stable if the % Change is less than 15% as per US FDA guidelines [13]. The stability of spiked human plasma samples stored at room temperature (bench top stability) was evaluated for 48 h. The stability of spiked human plasma samples stored at -30 °C in autosampler (autosampler stability) was evaluated for 55.5 h. The autosampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the autosampler at 20°C for 55.5 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the autosampler at 20°C for 27

h. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen at –30 °C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels were used for the freeze–thaw stability evaluation. For long term stability evaluation the concentrations obtained after 71days were compared with initial concentrations.
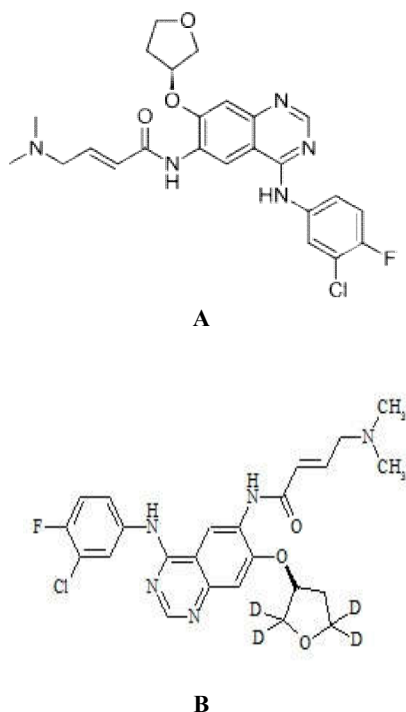

Fig.1. Chemical Structures of A) Afatinib (AT) B) Afatinib-D4 (ATD4)
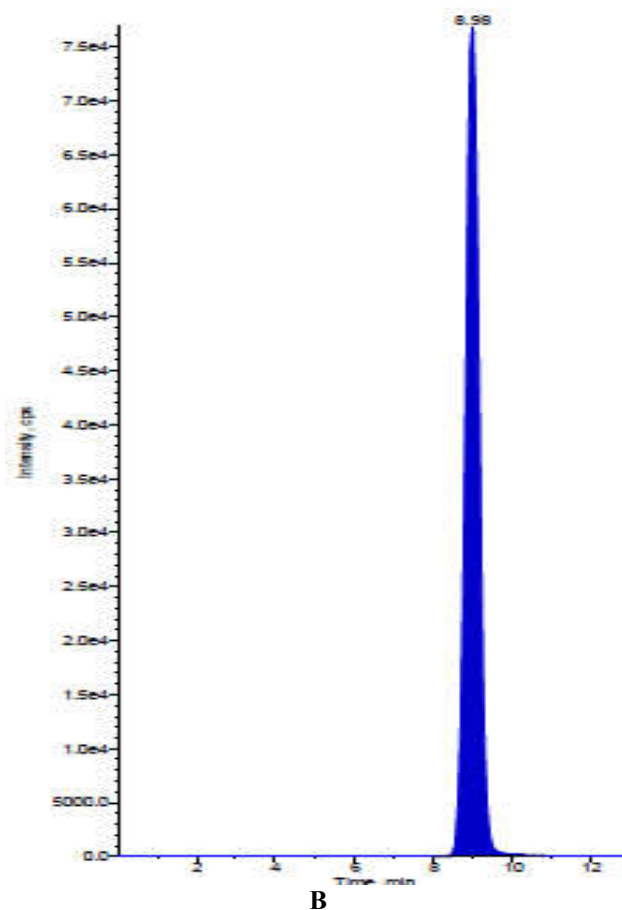

Figure 2. Chromatograms of A) Afatinib (AT) B) Afatinib -D4 (ATd4)

RESULTS AND DISCUSSION

Method development and validation

The goal of this work is to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of AT from plasma samples. LC-MS/MS has been used as one of the most powerful analytical tool in clinical pharmacokinetics for its selectivity, sensitivity and reproducibility. The MS optimization was performed by direct infusion of solutions of AT and AT d4 into the ESI source of the mass spectrometer. The vital parameters like ionization type, temperature, voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the respective productions from the protonated AT and AT d4 molecules. Chromatographic conditions, especially, composition of the mobile phase, selection of suitable column was optimized through several trials to achieve the best resolution and increase the signal of analyte and internal standard. Different extraction methods like solid phase extraction, Liquid-liquid extraction, precipitation methods were optimized for extraction of AT and AT d4 from the plasma sample. A good separation and elution were achieved using 5 mM ammonium formate: acetonitrile (10:90v/v) as the mobile phase, at a flow-rate of 0.6 mL/minutes and injection volume of 20 µL. Liquid-liquid extraction was chosen to optimize the drug and internal standard. The retention time was optimized 9.2 minutes for AT and 8.2 minutes for AT d4 (Figure 2).
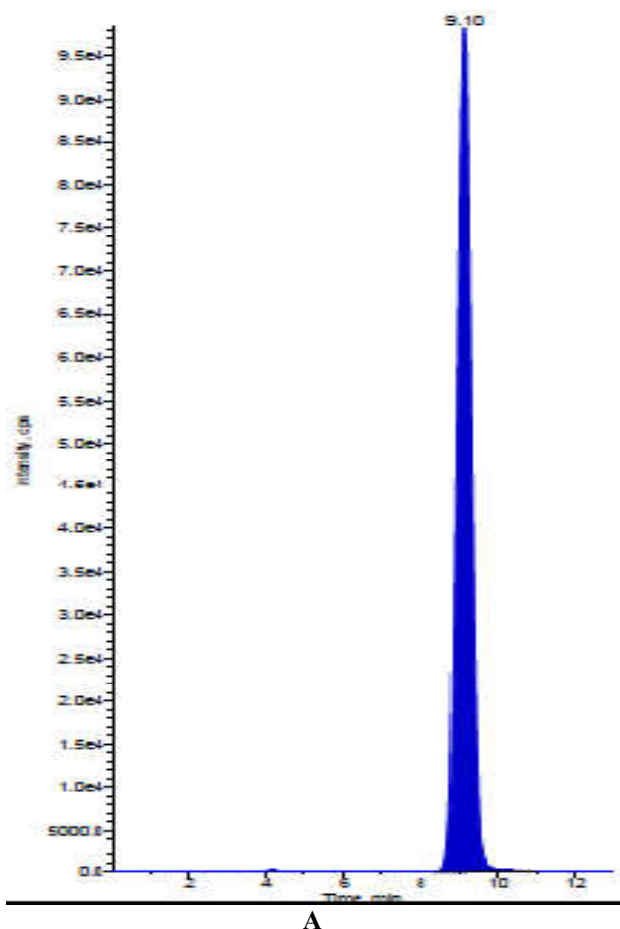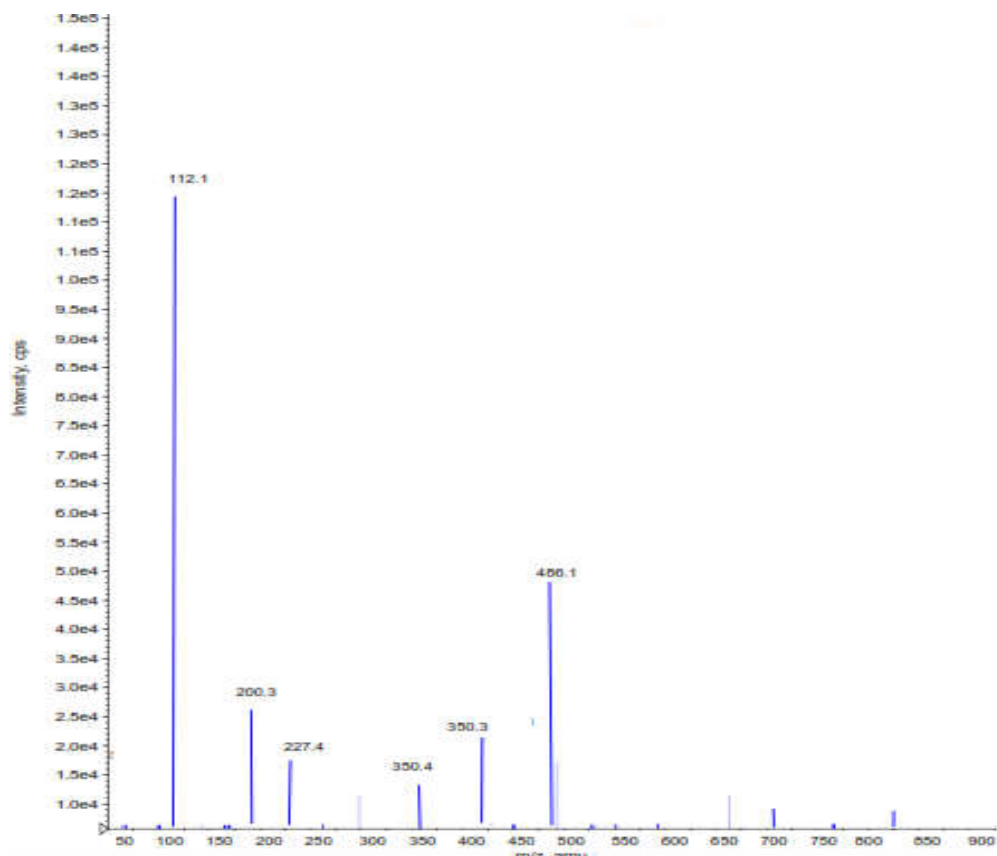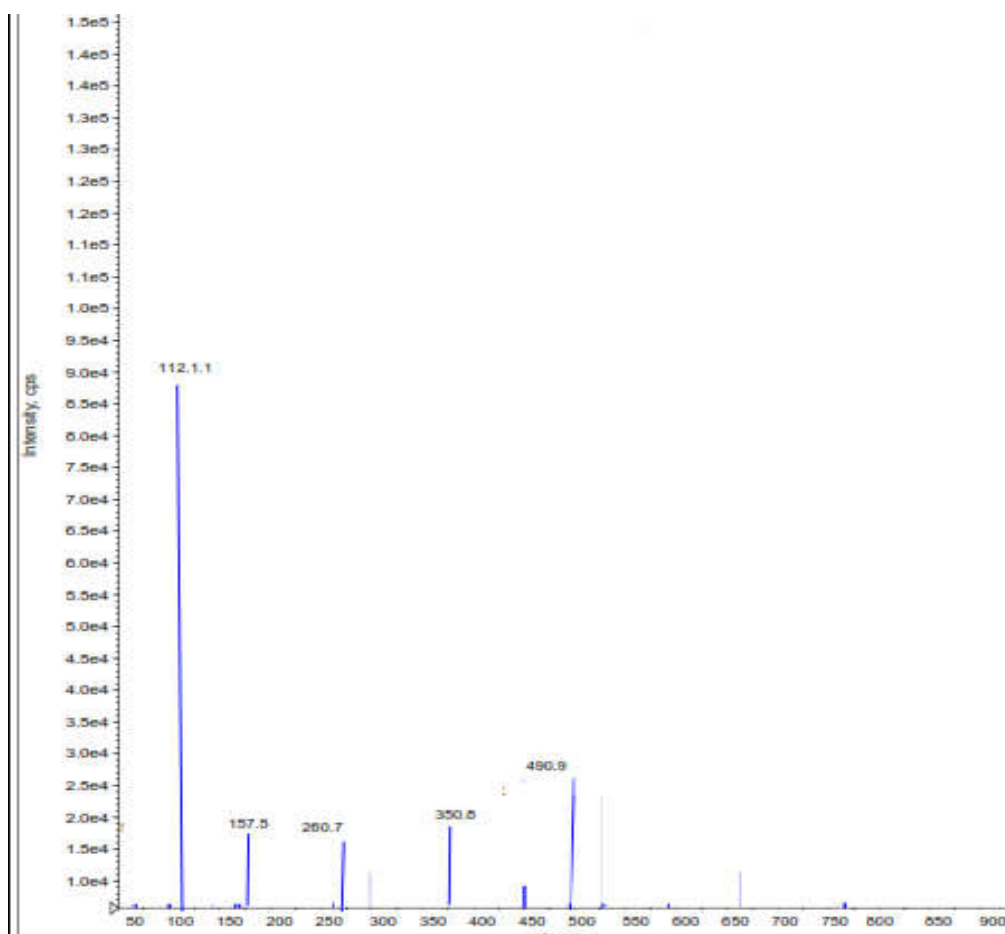
Figure 3. MRM scan of Afatinib (AT)

Figure 4. MRM scan of Afatinib -D4 (ATd4)

Table 1. Calibration curve details of Afatinib

Spiked plasma concentration (pg/mL)	Concentration measured (mean) (pg/mL) ($n = 5$)	Precision RSD. ($n = 5$)	Accuracy %
50.0	51.0 ± 1.3	2.5	102.00
100.0	96.6 ± 4.7	4.9	96.60
500.0	498.4 ± 24.7	5.0	99.68
1000.0	1000.0 ± 17.1	1.7	100.00
2000.0	2013.0 ± 74.6	3.7	100.65
4000.0	4008.4 ± 206.6	5.2	100.21
6000.0	5956.5 ± 190.7	3.2	99.28
8000.0	7952.2 ± 165.6	2.1	99.40
10000.0	10317.1 ± 487.6	4.7	103.17

Table 2. Precision and accuracy (analysis with spiked plasma samples at three different concentrations)

Spiked plasma concentration (pg/mL)	Within-run (n=6)			Between-run (n=6)		
	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD	Accuracy %	Concentration measured (pg/mL) (mean±S.D.)	Precision RSD	Accuracy %
50.00	51.4±2.3	4.19	105.57	55.5±4.1	7.08	114.75
150.00	154.9±3.4	2.36	106.1	157.9±5.7	3.46	106.44
3000.00	3103.8±102.0	7.41	112.76	3133.0±108.2	3.5	105.01
8000.00	7297.1±89.9	1.1	90.67	7278.7±275.5	3.8	92.78

Table 3. Stability of Afatinib in human plasma samples

Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (n=6) Mean ± SD	RSD (n=6) (%)	Accuracy (%)
Bench top in plasma	RT	150.0	148.3 ± 8.1	5.45	95.48
	48 hr	8000.0	6728.3±206.3	3.05	98.75
Processed (extracted sample)	Autosampler	150.0	162.3 ± 2.4	1.54	108.03
	55.5 hr	8000.0	7536.7±294.5	3.97	97.48
Freeze/Thaw stability	-30^{0}C	150.0	156.5 ± 4.0	2.53	104.53
	Cycle-3	8000.0	7381.7±173.4	2.34	93.61
Long-term stability in human plasma	- 30^{0}C	150.0	160.3±13.2	8.25	95.64
	71 days	8000.0	7450.0±229.1	3.12	90.88

Linearity

Calibration curve was plotted as the peak area ratio (AT/AT d4) versus (AT) concentration. Calibration was found to be linear over the concentration range of 50.0 – 10000.0 pg/mL. The correlation coefficient (r^2) was greater than 0.9997 for all curves (Table 1).

Selectivity

The selectivity of the method assessed by comparing chromatograms of blank plasma. There were no significant endogenous peaks were observed at respective retention time of AT and AT d4. The results indicate that the method exhibited both good specificity and selectivity.

Precision and Accuracy

Precision and accuracy for this method were controlled by calculating the Within-run and Between-run variations at three concentrations (150.0, 3000.0 and 8000.0 pg/mL) of QC samples in six replicates. As shown in Table 2 the Within-run Precision and Accuracy were between 1.1 to 7.4 and 90.67 to 112.76 % for AT. Similarly, the Between-run Precision and Accuracy were between 3.5 to 3.77 and 92.78 to 106.44 % for AT. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Matrix effect

The ion suppression/enhancement in the signal at MQC level was found % CV 1.21. These results indicating that there is no effect on ion suppression and ion enhancement.

Recovery

The %recoveries of AT were determined at three different concentrations 150.0, 3000.0 and 8000.0 pg/mL, were found to be 99.6 ±3.53, 88.2 ±2.7 and 97.60 ± 4.7 %. The overall average recoveries of AT and AT d4 was found to be 95.1 ± 6.1 and 98.1 ± 4.47%. Recoveries of the analyte and IS were consistent, precise and reproducible.

Limits of Detection and Quantification (LOD&LOQ)

The LOQ & LOD signal-to-noise (S/N) values found for six injections of AT at LOQ & LOD concentrations were 31.95pg/ml and 10.5pg/ml.

Stability (Freeze - thaw, Auto sampler, Room temperature, Long term)

Stock solution stability was performed to check stability of AT and ATd4 in stock solutions prepared in methanol and stored at 2-8 °C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 26 days. The % change for AT and ATd4 were -0.02% and 0.03% respectively indicate that stock solutions were stable at least for 26 days. Room temperature and autosampler stability for AT was investigated at LQC and HQC levels. The results revealed that AT was stable in plasma for at least 72 h at room temperature, and 78 h in an auto sampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with AT at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that AT was stable in a matrix up to 71days at a storage

temperature of -30 °C. The results obtained from all these stability studies are tabulated in Table 3. Precision (%CV) is less than 5 for Room temperature, long-term, Freeze thaw, auto sampler stability.

Conclusion

The proposed research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant advantages over other described methods in previously. Quantification of Afatinib was compared with respective isotope labeled internal standards. Extraction of analyte and IS were achieved by using LLE. Linearity range, column, mobile phase, flow rate, injection volume, plasma usage volume for analysis was improved. Hence this method has significant advantages over previously reported methods in-terms of Selectivity, sensitivity, Linearity, Reproducibility.

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