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Research article

A high performance LC-MS/MS method for the estimation of Erlotinib in human plasma

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Abstract

A High Performance Liquid Chromatography Mass Spectrometric Method for the estimation of Erlotinib in human plasma has been developed and validated using Erlotinib D6 as an internal standard (IS). The extraction of analyte and IS was accomplished by Liquid-Liquid Extraction technique. The method has been validated over a concentration range of 1.00ng/mL to 2502.02 ng/mL. Chromatographic separations was achieved using Waters X Bridge C18, 75mm x 4.6mm, 3.5 μ , column eluted at flow rate of 0.8 mL/minute with 1:1 splitted post column with mobile phase Acetonitrile: Ammonium acetate buffer pH 9.2 (70:30 v/v). The overall run time of method was about 3.0 min. with elution times of Erlotinib and its internal standard Erlotinib D6 at around 1.3 min. The multiple reaction monitoring transitions were set at 394.2 > 278.1 (m/z) and 400.4 > 278.1 (m/z) for Erlotinib and Erlotinib D6 respectively. The calibration curves were linear ($r^2 \geq 0.99$) over the range of 1.0-2502.02 ng/mL with lower limit of quantitation validated at 1.0 ng/mL. Extraction recoveries were > 80 % for both Erlotinib and its stable labeled IS Erlotinib D6. The within run and between run precisions were within 0.62%-7.07%, while accuracy ranged from 94.4 to 103.3%.

Introduction

Erlotinib (TARCEVA) N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine]] is a human HER1/EGFR tyrosine kinase inhibitor. The molecular formula is C₂₂H₂₃N₃O₄ and molecular weight is 393.2. Abnormal activation of specific protein tyrosine kinases has been demonstrated in many human neoplasms, making them attractive molecular targets for cancer therapy. It is indicated for treatment of patients with locally advanced or metastatic non-small cell lung cancer [1-3]. Erlotinib is ~60% absorbed after oral administration and its bioavailability is increased to almost 100% by food. Peak plasma levels occur 4 hours after an oral dose. The solubility of erlotinib is pH dependent. Erlotinib solubility decreases as pH increases. Following absorption, erlotinib is ~93% protein-bound to albumin and α_1 -acid glycoprotein. Its t_{1/2} is ~36 hours. Erlotinib is metabolized primarily by CYP3A4 and to a lesser extent by CYP1A2 and CYP1A1. Cigarette smoking has been shown to reduce erlotinib AUC. Drugs that alter the pH of the upper GI tract may alter the solubility of erlotinib and

reduce its bioavailability [4-7]. The most common adverse reactions in patients receiving erlotinib were diarrhea and rash. Serious interstitial lung disease also has been reported. Other adverse effects include elevated liver enzymes and bleeding, especially in patients receiving warfarin. Co-administration of CYP3A4 inhibitors is expected to increase toxicity to erlotinib.

Earlier publications have described methods for determination of erlotinib in biological matrix using high performance liquid chromatography (HPLC) methods with ultraviolet detection (UV) [8-10], and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods [11-17]. In this study, we attempted to develop a simple and rapid method for the determination of erlotinib in human plasma using a HPLC MS/MS method to evaluate the oral pharmacokinetics of erlotinib tablets. Compared with previous methods, the present method has the following advantages: less plasma was required, sample preparation was simpler, and the analysis time was shorter using normal HPLC conditions making it suitable to analyze large number of samples.

Experimental

Solvents and reagents

The reagents/materials used during analysis includes Ammonium acetate (AR Grade), Methanol (HPLC Grade), Methyl tertiary butyl ether (AR Grade), Liquor Ammonia 25% (GR Grade), Acetonitrile (HPLC Grade), Milli-Q water.

Erlotinib and Erlotinib D6 with purity >98% were purchased from Clearsynth Bangalore., (Figure 1a and 1b). Blank human K₂EDTA plasma was purchased from blood bank.

Erlotinib Stock Solution

Approximately 5 mg of erlotinib was weighed and transferred to 5 ml volumetric flask. It was dissolved and made up to the mark with HPLC grade methanol to make approximately 1.0 mg/ml stock solution. This stock solution was transferred in a reagent bottle with appropriate label and stored at 2°C to 8°C. This solution was used within 7 days from the date of preparation. Further dilutions of erlotinib were prepared in Methanol: MilliQ water, 50:50 v/v for spiking into plasma.

Erlotinib D6 (IS) Stock Solution

Approximately 2 mg of erlotinib D6 was weighed and transferred to 2 ml volumetric flask. It was dissolved and made up to the mark with HPLC grade methanol to make approximately 1.0 mg/ml stock solution. This stock solution was transferred in a reagent bottle with appropriate label and stored at 2°C to 8°C. This solution was used within 7 days from the date of preparation. Further stock dilutions of erlotinib D6 was prepared in Methanol: MilliQ water, 50:50 v/v solution.

Ammonium acetate buffer (pH 9.2 ± 0.05)

About 38.5 mg of Ammonium acetate was weighed and transferred into 100 ml volumetric flask. It was dissolved with Milli-Q water / HPLC grade water and volume was made up to the mark. It was then sonicated in an ultrasonicator for 5 to 10 minutes. The pH was adjusted to (9.2 ± 0.05) with diluted ammonia solution. The buffer was used within 2 days from the date of preparation.

Rinsing Solution

A mixture of acetonitrile and Milli-Q water was prepared in the volume ratio of 70:30 v/v as rinsing solution. It was sonicated in an ultrasonicator for 5 to 10 minutes. The solution was used for auto sampler rinsing to avoid carryover.

Mobile Phase

A mixture of acetonitrile and ammonium acetate buffer pH (9.2 ± 0.05) was prepared in the volume ratio of 70:30 v/v as mobile phase. It was sonicated in an ultrasonicator for 5 to

10 minutes. This was used within 4 days from the date of preparation.

Calibration Curve Standards and Quality Control Samples

Calibration curve standards of Erlotinib concentrations ranging from 1.0 ng/ml to 2502.02 ng/ml were prepared by spiking appropriate dilutions of working stock solution in pooled blank plasma having K₂EDTA as anticoagulant. The five levels of QC samples of Erlotinib concentrations ranging from 1.0 ng/ml (LLOQ QC), 2.81 ng/ml (LQC), 510.90 ng/ml (M1QC), 1021.80 ng/ml (MQC) and 2043.60 ng/ml (HQC) were prepared by spiking appropriate dilutions of stock solution in pooled blank K₂EDTA plasma. Spiking solution was not used more than 5 % of total plasma volume. These samples were stored in deep freezer below –50°C until use.

Plasma sample extraction

The spiked plasma samples were retrieved from the deep-freezer and thawed in a water bath at room temperature. The samples were extracted using Liquid-Liquid Extraction technique. The thawed samples were vortexed to ensure complete mixing of the contents. A 50 µl of IS dilution mixture (approximately 4000.0 ng/ml of Erlotinib D6) was taken in pre-labeled polypropylene tubes except in blank samples wherein 50 µl of dilution solution was added to compensate. A 200 µl of sample was added to it and vortexed. This was followed by addition of 200 µl of 5% ammonia solution and vortexed again followed by addition of 2 ml methyl tertiary butyl ether (MTBE) and further vortexed for about 2 minutes. It was centrifuged for 2 min at about 4090 rpm maintained at temperature 5°C. After centrifuge 1 ml of supernatant was taken out into pre labeled glass tubes. The supernatant was dried at 50°C under a stream of nitrogen. The dried residue was reconstituted with 0.5 ml of mobile phase and transferred into disposable auto sampler glass vials. Samples were analyzed on AB Sciex API 4000 LC-MS/MS system using Turbo ion spray in positive mode.

Equipment

Shimadzu HPLC equipped with dual pump, auto sampler and Column Oven. Mass spectrometer AB SCIEX API 4000 LC-MS/MS and data acquisition system Software Analyst Version 1.6.2 were used for the quantitative determination. The chromatograms were acquired using software Analyst version 1.6.2. The slopes, intercepts and goodness of fit were determined by linear regression analysis using the ratios of analyte/IS peak areas of the calibration curve standards. A weighting factor of 1/x² (1/ concentration²) was used in the calculation of the linear regression line and the concentrations of QC samples were calculated.

Chromatographic Conditions

Analytes were separated on a Waters XBridge C18, 75mm x 4.6mm, 3.5 μ , column maintained at temperature of 40°C. An isocratic flow-rate of 1.0 ml/minute with 1:1 splitted post column with mobile phase Acetonitrile: Ammonium acetate buffer (70:30 v/v) was used for chromatographic separation. The pH of ammonium acetate buffer was adjusted to (9.2 $\pm$ 0.05) with diluted liquor ammonia solution. Following 5 μ l injection analytes were separated. The overall run time was 3.0 min. A mixture of acetonitrile and Milli-Q water in the volume ratio of 70:30 was used as needle wash solution to avoid carry over between two injections.

Mass Spectrometry

Mass spectrometric detection was performed on an Applied Biosystems MDS Sciex (Concord, Ontario, Canada) API 4000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. ESI ionization was

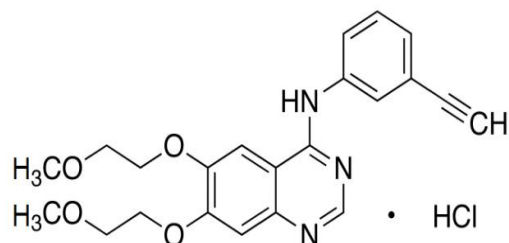


Figure 1a. Chemical structure of Erlotinib.HCl

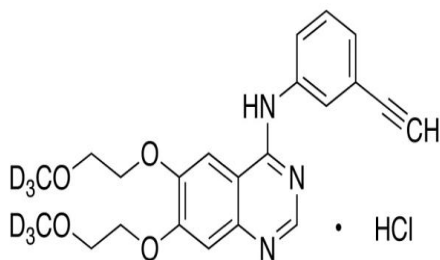


Figure 1b. Chemical structure of Erlotinib D6.HCl

performed in the positive ion mode. The tandem mass spectrometer was operated at unit resolution in the selected reaction monitoring mode (SRM). The multiple reaction monitoring transitions were set at 394.2 > 278.1 (m/z) and 400.4 > 278.1 (m/z) for Erlotinib and Erlotinib D6 respectively (Figure 2a, b, c, d).

The mass spectrometric conditions were optimized for Erlotinib and Erlotinib D6 by continuous infusion of the standard solution at the rate of 10 μ l min⁻¹ using a Harvard infusion pump. The ion source temperature was maintained at 550°C. The ion spray voltage was set at 4000 V. The curtain gas (CUR) was set at 50 and the collision gas (CAD) at 12. The optimal collision energy (CE) was 15 V. The following parameters of ion path were used as the most favorable ones: de-clustering potential (DP) at 60 V and entrance potential (EP) at 45 V. Focusing potential (FP) was maintained at 10V.

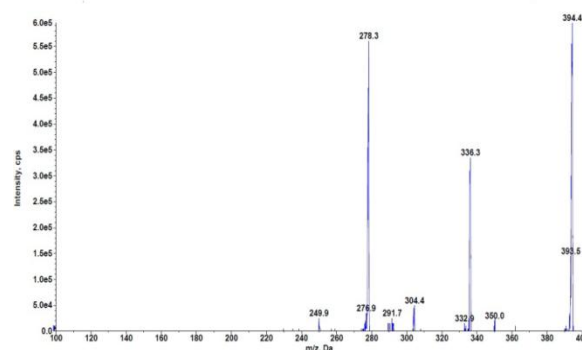


Figure 2 b Product ion spectra of Erlotinib

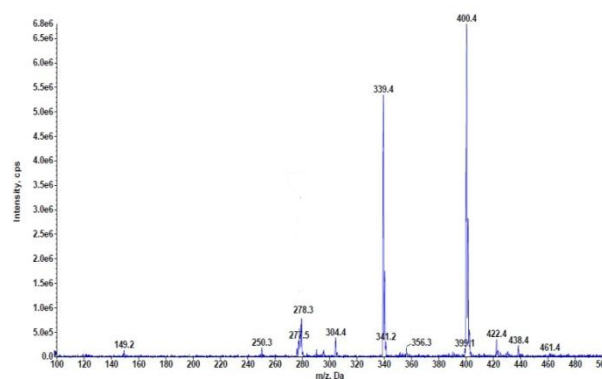


Figure 2c. Mass spectra of Erlotinib D6

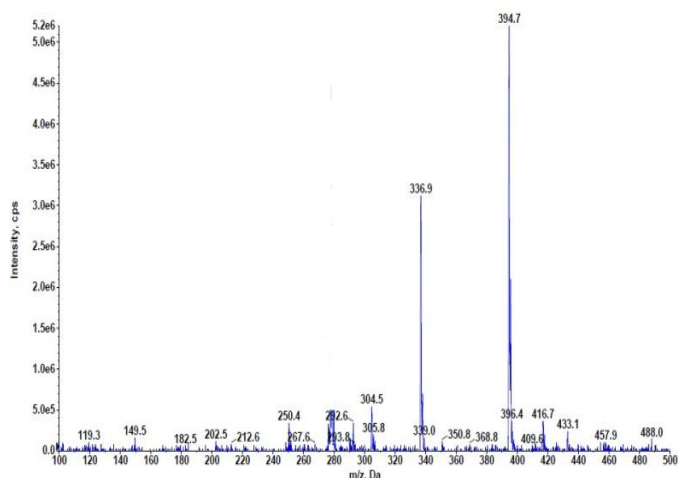


Figure 2a. Mass spectra of Erlotinib

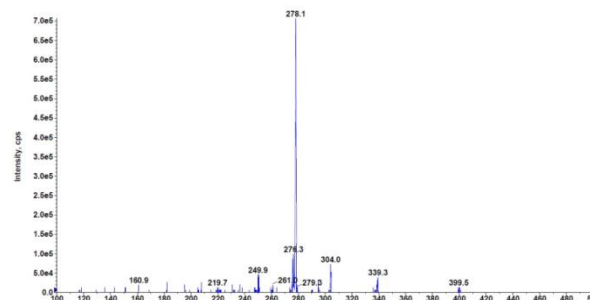


Figure 2d. Product ion spectra of Erlotinib D6

The quantification was performed via peak area ratio. Data acquisition and processing were accomplished using the Applied Biosystems Analyst version 1.6.2 software. Calibration curves were generated using peak area ratios of the components to internal standards versus the known concentrations with a linear regression equation of $1/\text{concentration}^2$.

$$y = mx + b$$

Where, y = peak area ratio of Erlotinib to Erlotinib D6 (IS), m = slope of the calibration curve, x = concentration of Erlotinib in ng/mL, b = y-axis intercept of the calibration curve.

Method Validation

The HPLC-MS/MS method was validated in accordance with the Guidance for industry, Bioanalytical method validation, as specified by US FDA [18].

The potential presence of endogenous contaminating compounds that may interfere with the analytical assay was determined by analyzing blank human K₂EDTA plasma samples of ten different individual lots. Representative chromatogram of extracted blank plasma sample is given in (Figure 3a- b). No significant interference from endogenous components was observed at retention time of analyte and IS in all the human plasma batches screened.

Sensitivity

The lowest limit of quantification (LLOQ) was set at the concentration of 1.0 ng/ml (Figure 3c). Six replicates of blank plasma were spiked at a concentration of 1.0 ng/ml. The precision and accuracy at LLOQ were found to be 2.42 % and 103.3 % respectively.

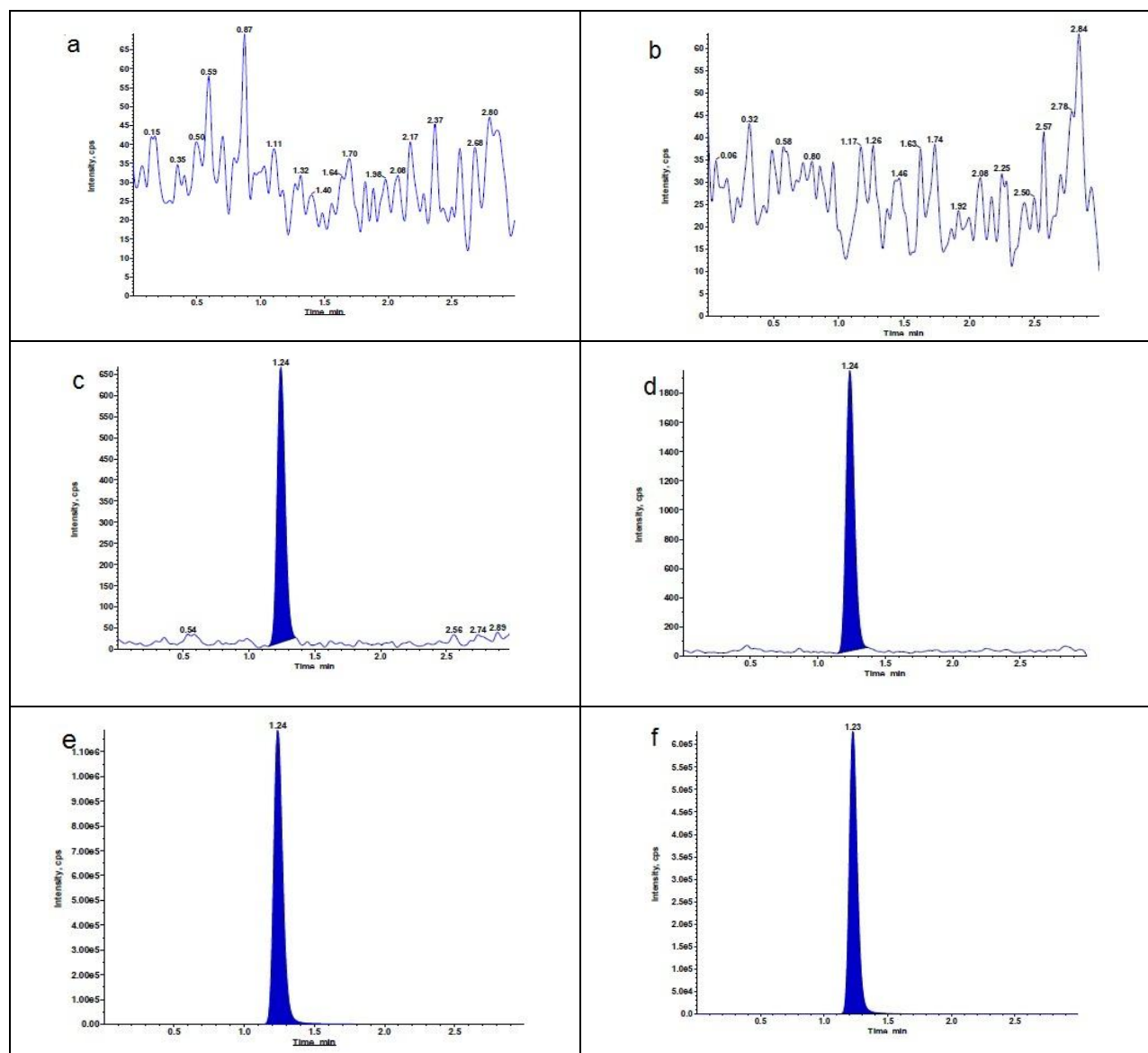


Figure 3. Representative chromatograms a. Blank (Erlotinib), b. Blank IS (Erlotinib D6), c. Extracted LLOQ 1.0 ng/ml, d. Extracted LQC 2.81 ng/ml, e. Extracted HQC 2043.6 ng/ml, f. Extracted IS Erlotinib D6.

Matrix Factor

Ten different lot of blank plasma were taken, extracted as per extraction method described above and spiked with LQC and HQC equivalent aqueous concentrations after extraction, thus achieving unextracted samples. Responses of these unextracted samples were compared with spiked extracted LQC and HQC samples to calculate matrix factor. No significant matrix effect was observed at low and high concentration levels

Recovery

The peak areas of extracted LQC, MQC and HQC samples of Erlotinib and peak areas of extracted MQC samples of IS Erlotinib D6 were compared against the peak areas of respective Unextracted QC samples. The overall mean recovery of Erlotinib and IS Erlotinib D6 was found to be 80.1 % and 91.7 % respectively.

Results and Discussion

Linearity, Precision and Accuracy

A regression equation with a weighing factor of $1/(\text{concentration}^2)$ was judged to produce the best fit for the concentration-detector response relationship for Erlotinib in human plasma. The representative calibration curve for regression analysis is illustrated in Figure 4. Coefficient of determination (r^2) was greater than 0.996 in the concentration range from 1.0 ng/ml to 2502.02 ng/ml (Table 1).

Table 1. Back calculated concentrations of calibrators for Erlotinib

Nominal Concentration of Erlotinib	Back calculated concentration (mean $\pm$ SD) n=3	RSD (%)	Accuracy (%)
1.00	1.00 $\pm$ 0.0000	0.00	100.00
2.64	2.643 $\pm$ 0.0153	0.58	100.11
12.56	12.780 $\pm$ 0.2364	1.85	101.75
59.81	63.320 $\pm$ 1.2550	1.98	105.87
239.26	248.287 $\pm$ 8.3560	3.37	103.77
957.02	975.113 $\pm$ 16.9966	1.74	101.89
2126.72	2020.580 $\pm$ 18.2153	0.90	95.01
2502.02	2299.127 $\pm$ 59.3594	2.58	91.89

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ QC, LQC, M1QC, MQC and HQC samples respectively, during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ QC, LQC, M1QC, MQC and HQC samples to their respective nominal values, expressed in percentage (Fig. 4). Within batch precision ranged from 0.62 % to 3.50 % and the within batch accuracy ranged from 96.29 % to 103.3 %. Intra-day precision ranged from 1.25 % to 6.33 % and the intra-day accuracy ranged from 95.33 % to 103.8 %. Between batch/Inter day precision

ranged from 1.29 % to 6.23 % and the between batch/Inter day accuracy ranged from 95.44 % to 103.2 %. (Table 2)

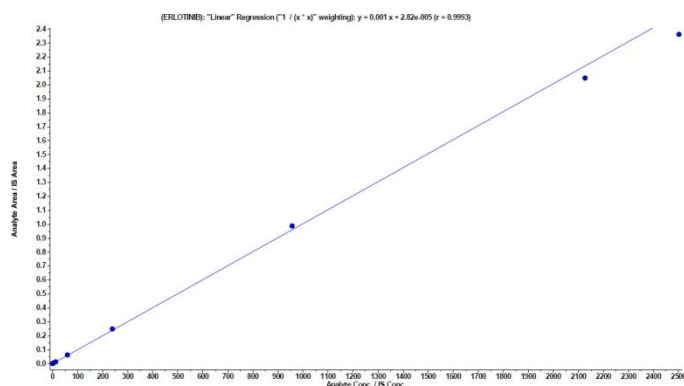


Figure 4. Erlotinib linearity curve over the range of 1.0 ng/ml to 2502.02 ng/ml

Stability Studies

Standard stock solution stability

Room temperature stock solution stability of Erlotinib: The standard stock solution of Erlotinib was prepared. Stock solution was kept on the bench at room temperature for 24 hrs. Dilution was prepared from stock solution as stability stock and a fresh dilution was prepared as comparison stock. Room temperature stock solution stability of Erlotinib was carried out by injecting six replicates from the above prepared stock dilutions of Erlotinib at room temperature. The stability was found to be 98.21%.

Short Term Room Temperature (Bench top) Stability for 6 hrs

Short-term room temperature stability was determined by using six sets of QC (LQC and HQC) samples at room temperature. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. Erlotinib was found to be stable up to 6 hrs. The precision ranged from 1.95 % to 4.48 % and the accuracy ranged from 94.82 % to 105.69 %. (Table 3)

Auto sampler stability for 75 hrs

In assessing the auto sampler stability, six sets of QC samples (LQC and HQC from PA Batch: 01) were processed and placed in the auto sampler. They were injected after a period of 75 hrs. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The results demonstrate that the processed samples were stable up to 51 hrs. The precision ranged from 1.88 % to 2.06 % and accuracy ranged from 95.82 % to 97.86 %. (Table 3)

Freeze-Thaw Stability

The stability of Erlotinib in human plasma was determined during 3 freeze-thaw cycles. Six sets of QC (LQC & HQC) samples were analyzed after FT-3 cycles. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the freeze-thaw QC samples and quantified against the freshly spiked CC standards. The precision ranged from 1.92 % to 4.58 % and accuracy ranged from 96.85 % to 102.56 %. The freshly spiked CC standards and QC samples were found within the acceptance criteria. (Table 4)

Long Term Stability data (below -50°C) for 156 days

The stability of Erlotinib, for plasma samples stored below -50°C was generated for 156 days by quantifying six sets of QC samples (LQC and HQC). Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The

precision of the calculated concentrations of QC samples ranged from 1.89 % to 2.10 % and accuracy ranged from 94.73 % to 104.98 %. The freshly spiked CC standards and QC samples were found within the acceptance criteria. (Table 4)

Dilution Integrity

Dilution integrity samples were prepared by spiking about 1.6 times the highest standard concentration of Erlotinib (2502.02 ng/ml). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. These dilution QC samples were analyzed along with CC standards and were calculated using 2x and 4x dilution factor respectively. The precision and accuracy for a dilution factor of 2 was found to be 1.33 % and 95.16 % respectively. Similarly, the precision and accuracy for a dilution factor of 4 were found to be 1.94 % and 98.00 % respectively (Table 5).

Table 2. Within-day and between days accuracy (% of nominal concentration) and precision (% RSD) of Erlotinib in human plasma

QC i.d	QC Nominal Concentration (ng ml ⁻¹)	Within Batch (n=6)	
		Precision (RSD, %)	Mean Accuracy (%)
LLOQ	1.0	2.42	103.3
Low	2.81	3.50	102.42
Mediuml	510.9	0.62	103.28
Medium	1021.8	1.22	99.99
High	2043.60	0.78	96.29

QC i.d	QC Nominal Concentration (ng ml ⁻¹)	Within same day (n=12)		Different days (n=18)	
		Precision (RSD, %)	Mean Accuracy (%)	Precision (RSD, %)	Mean Accuracy (%)
LLOQ	1.0	6.33	99.5	6.23	98.8
Low	2.81	4.00	101.64	3.71	100.93
Mediuml	510.9	1.65	103.8	1.69	103.2
Medium	1021.8	1.32	99.94	1.78	100.56
High	2043.60	1.25	95.33	1.29	95.44

Table 3. Bench top and post preparative (auto injector) stability

QC i.d	QC Nominal Concentration (ng mL ⁻¹)	Stability after 16 h at room temperature (Bench top stability, n=6)			Stability after 51 h at 10°C (post preparative, n=6)		
		Mean Concentration found (ng ml ⁻¹)	Precision (RSD, %)	Mean Accuracy (%)	Mean Concentration found (ng ml ⁻¹)	Precision (RSD, %)	Mean Accuracy (%)
Low	2.81	2.970	4.48	105.69	2.81	2.06	97.86
High	2043.60	1937.838	1.95	94.82	2043.60	1.88	95.82

Table 4. Long term and freeze thaw stability of Erlotinib

QC i.d	QC Nominal Concentration (ng ml ⁻¹)	Stability after three freeze thaw cycles			Long term stability of Erlotinib at -50°C after 156 days		
		Mean Concentration found (ng ml ⁻¹)	Precision (RSD, %)	Mean Accuracy (%)	Mean Concentration found (ng ml ⁻¹)	Precision (RSD, %)	Mean Accuracy (%)
Low	2.81	2.882	4.58	102.56	2.81	1.89	104.98
High	2043.60	1979.262	1.92	96.85	2043.60	2.10	94.73

Table 5. Table Dilution Integrity

Sample ID	Nominal Concentration (ng ml ⁻¹)	Mean Back Calculated Concentration n=6 (ng ml ⁻¹)	Precision (RSD, %)	Mean Accuracy (%)
2xDilution	4063.98	3867.18	1.33	95.16
4xDilution	4063.98	3982.79	1.94	98.00

Conclusion

In this manuscript, we described a sensitive and selective high performance liquid chromatography–tandem mass spectrometry method for the analysis of erlotinib in human plasma. Validation of the method in selected conditions shows that the method is selective and precise with linear response of mass spectrometer. The Liquid-Liquid Extraction procedures produced clean chromatograms and high and reproducible recovery was obtained for investigated compound. The method has been found suitable to support pharmacokinetic studies.

Conflict of Interest

Conflict of Interest: The authors declare that they have no conflict of interest

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