Hplc-Ms/Ms Method Development And Validation For Determining Stability of Brigatinib In Human Plasma Samples

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Abstract: The validated protein precipitation method was applied for estimation of brigatinib in human plasma with brigatinib-D6 as an internal standard (ISTD) by using HPLC-ESI-MS/MS. The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v) using the C18 column Ascentis Express (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The mass transitions of brigatinib and brigatinib-D6 obtained were m/z 570.3 → 484.6 and 585.1 → 484.6. The standard curve shows correlation coefficient (r²) greater than 0.9983 with a range of 5.00-10000.00 pg/ml using the linear regression model.

Keywords: Brigatinib; Human plasma; HPLC-ESI-MS/MS; Bioanalysis

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I. INTRODUCTION

Disease progression in patients with anaplastic lymphoma kinase-positive (ALKp) non–small cell lung cancer (NSCLC) treated with the first-generation tyrosine kinase inhibitor (TKI) crizotinib, or the second-generation inhibitors ceritinib and brigatinib, is often associated with secondary resistance mutations in ALK, or relapse in the brain. Where as, brigatinib, a selective ALK inhibitor with substantially more potent (12-fold) activity against ALK than crizotinib, in vitro [1-11]. Brigatinib is a multi-targeted tyrosine-kinase inhibitor useful for the treatment of non-small cell lung cancer (NSCLC) and other diseases, it is a potent inhibitor of ALK (anaplastic lymphoma kinase) and is in clinical development for the treatment of adult patients with ALK-driven NSCLC. It was granted orphan drug designation by the U.S. Food and Drug Administration (FDA) in May 2016 for the treatment of certain subtypes of non-small cell lung cancer (NSCLC) [12-16]. Brigatinib received breakthrough therapy designation from the FDA in October 2014 for the treatment of patients with ALK+ NSCLC whose disease is resistant to crizotinib. Both designations were based on results from an ongoing Phase 1/2 trial that showed anti-tumor activity of brigatinib in patients with ALK+ NSCLC, including patients with active brain metastases [17-20]. It inhibits autophosphorylation of ALK and ALK-mediated phosphorylation of the downstream signaling proteins STAT3, AKT, ERK1/2, and S6 in in vitro and in vivo assays. Brigatinib also inhibited the in vitro proliferation of cell lines expressing EML4-ALK and NPM-ALK fusion proteins and demonstrated dose-dependent inhibition of EML4-ALK-positive NSCLC xenograft growth in mice [21-25]. The chemical name for brigatinib is 5-chloro-N4-[2-(dimethylphosphoryl)phenyl]-N2-{2-methoxy-4-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]phenyl}pyrimidine-2,4-diamine. The molecular formula is C29H39ClN7O2P which corresponds to a formula weight of 584.10 g/mol. Brigatinib has no chiral centers. Brigatinib is an off-white to beige/tan solid. The pKas were determined to be: 1.73 ± 0.02 (base), 3.65 ± 0.01 (base), and 8.04 ± 0.01 (base) [26]. The literature survey reveals that, very few methods were reported on the for quantification of brigatinib by using UPLC-MS/MS [27]. There is no method reported for estimation of brigatinib using deuterated internal standard in biological samples. The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for quantitative determination of brigatinib in human plasma by HPLC-ESI-MS/MS.

II. MATERIALS AND METHODS

2.1 Materials:
2.1.1 Chemical Resources

Brigatinib (BT) and brigatinib-D6 (BTD6) (VARDA Biotech, Mumbai, India), methanol and acetonitrile (J.T Baker, USA), formic acid (Merck, Mumbai, India), Ultra pure water (Milli-Q system, Millipore, Bedford, MA, USA), human plasma (Doctors pathological labs, hyderabad, India). The chemicals and solvents were used in this study analytical and HPLC grade.
Instrument Resources

An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

Methods

Chromatographic condition

The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v), gave the best peak shape and low baseline noise was observed using the Ascentis Express C18 (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 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 10 µl for better ionization and chromatography.

Detection

The pure drug of BT and BTD6 were prepared in methanol (10.00 ng/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 570.3→484.6 and 585.1→484.6 for BT and BTD6. The mass fragmentation pattern of parent and product ions mass spectras were depicted in figure 2.

Standard calibration and quality control samples preparation

Stock solutions of BT (1000.00 µg/ml) and BTD6 (1000.00 µg/ml) were prepared in methanol. The internal standard (BTD6) spiking solution (500.00 ng/ml) was prepared in 75% methanol from BTD6 stock solution. Stock solutions of BT, BTD6 and intermediate spiking solutions were stored in refrigerated conditions (2-8°C) until analysis. Calibration standards (5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml), quality control samples of lower limit QC, low QC, mid QC, high QC (5.00, 15.00, 3000.00, 7000.00 pg/ml) were used by spiking the appropriate amount of standard solution in the drug free plasma and stored at -30°C till analysis.

Sample extraction

The protein precipitation method was applied to extract BT and BTD6. To each labelled polypropylene tube 50 µl of BTD6 (500.00 ng/ml) was mixed with the 100 µl plasma sample, then 0.25 ml of acetonitrile were added, vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 20°C. The organic phase was transferred to auto sampler vials containing 100 µl of 0.1% formic acid and injected into the HPLC-ESI-MS/MS for analysis.

Method validation

The developed method was validated over a linear concentration range of 5.0–10000.0 ng/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section [28–30].

Selectivity and Specificity

Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of AT retention time and less than 5% for BTD6 retention time.

Limit of Quantification (LOQ)

Six LLOQ standards were prepared in screened plasma lot along with IS and signal to noise ratio (S/N) was calculated using analyst software.

Linearity

Calibration standards were prepared to obtain linearity range of 5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml and assayed in five replicates on five different days.

Precision & Accuracy

One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (5.00 pg/ml), Low QC (15.00 pg/ml), Mid QC (3000.00 pg/ml) and High QC
(7000.00 pg/ml) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix Effect
Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (3000.00 pg/ml) and compared with un-extracted standards of the same concentration.

Recovery
The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (15.00 pg/ml), medium (300.00 pg/ml), high (7000.00 pg/ml).

Stability studies

Bench top Stability (Room Temperature Stability, 24 h)
Six replicates of spiked low and high concentrations (Bench top stability samples) were set aside at ambient temperature up to 24 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Freeze and thaw stability (after 3rd cycle at -30°C)
Six replicates of low and high concentrations (FT stability samples) were frozen at -30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler stability (2-8°C, 65 h)
Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 65 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-30°C, 45 Days)
After completion of the stability period stored at -30°C (45 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

III. RESULTS AND DISCUSSION

3.1 Method development
On the way to develop a simple and easy applicable method for determination of BT in human plasma, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results. Separation of the BT was performed with different branded RP-HPLC C18 columns. Initial separation was performed with isotropic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of 0.1% acetic acid: acetonitrile (20:80 v/v) and 0.1%acetic acid: methanol (20:80 v/v) gave the best response, but poor peak shape was observed. Some series of trials a mobile phase consisting of 0.1% formic acid in combination with methanol and acetonitrile in varying combinations were tried. Using a mobile phase containing 0.1% formic acid in combination with methanol (25:75 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Express C18 (50 mm × 4.6 mm, 2.7 μm) analytical column with a flow rate of 0.6 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 40 °C and temperature of auto sampler was maintained at 4°C. Injection volume of 10 μl sample was adjusted for better ionization and chromatography. For selection of internal standard, Afatinib Dimaleate, Imatinib Mesylate and Lenvatinib Mesylate were tried with optimized mobile phase and column conditions. Finally brigatinib-D6 (BTD6) was selected as internal standard in terms of better chromatography and extractability.

The retention times of analyte (BT) and internal standard (BTD6) were eluted at 1.42 ± 0.2 min and 1.44 ± 0.2 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the PPT was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard. Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 μl/min. Brigatinib gave more response in positive ion mode as compare to the negative ion mode.

www.irjes.com 51 | Page
To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for Brigatinib and BrigatinibD8, respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for Brigatinib and BrigatinibD8.

The predominant peaks in the primary ESI spectra of BT and BTD6 correspond to the MH ions at m/z 570.3 and 585.1 respectively. Productions of BT and BTD6 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 484.6 for both respectively. The parent and productions mass spectrums of BT and BTD6 were shown in Figure 2 & 3.

3.1 Method validation

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed at retention times of BT and BTD6 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.0 ng/ml. Represent chromatograms were shown in Figure 4.

Linearity

Linearity was plotted as a peak area ratio (BT peak area / BTD6 peak area) on the y-axis against BT concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for BT over a linearity range of 5 to 10000.00 pg/ml. The correlation coefficient was greater than 0.9980 for BT. The %CV was less than 15% and mean %accuracy was ranged between 99.40 - 102.67%. Results were presented in Table 1.

Precision & Accuracy

Intra and inter batch %accuracy for AT was ranged between 94.17-96.00 and 91.66 to 99.34. %CV is 2.16 to 5.64 and 1.64% - 8.00%.

Recovery

The mean %recovery for LQC, MQC, HQC samples of BT were 99.85%, 95.30% and 93.54% respectively. The overall mean %recovery and %CV of BT across QC levels is 96.23% and 3.38%. For the BTD6 (internal standard) the mean % recovery and %CV is 91.68% and 7.09%.

Matrix Effect

No significant matrix effect found in different sources of rat plasma tested for BT, BTD6. The %CV was found to be 3.71.

Stability (freeze–thaw, auto sampler, bench top, long term)

Quantification of the BT in plasma subjected to three freeze–thaw cycles (~30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

IV. CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 5.0–10000.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 6.0% and %accuracy ranged from 98.9%–102.4%. The overall %recovery for BT, BTD6 was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid protein precipitation extraction with less run time of 3.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Brigatinib.

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Conflict Of Interest

Authors declare that, there is no conflict of interest.
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**Figures**

(A)  
(B)  
Fig.1: Chemical structures of A) Brigatinib B) Brigatinib-D6
**Fig. 2** - Mass fragmentation pattern of Brigatinib (BT)

**Fig. 3** - Mass fragmentation pattern of Brigatinib D6 (BTD6)
Fig. 4 - Representative chromatograms of Brigatinib in plasma a) Blank plasma chromatogram for interference free BT and BTD6 b) Chromatogram of LLOQ sample (BT with BTD6).

| Tables |
| Table. 1 - Calibration curve details |
| Spiked plasma Concentration (pg/ml) | Concentration measured (pg/ml) (Means±D) | %CV (n=5) | %Accuracy |
| 5.00 | 4.99±0.01 | 1.4 | 99.9 |
| 10.00 | 10.24±0.02 | 3.6 | 101.7 |
| 50.00 | 49.89±0.15 | 3.3 | 100.1 |
| 100.00 | 100.24±0.22 | 2.5 | 100.1 |
| 500.00 | 501.6±0.27 | 3.8 | 100.1 |
| 1000.00 | 1004.22±0.21 | 2.6 | 101.7 |
| 2000.00 | 1999.18±1.02 | 3.1 | 99.4 |
| 4000.00 | 4001.35±1.10 | 3.4 | 101.7 |
| 6000.00 | 6003.76±1.11 | 1.7 | 102.6 |
| 8000.00 | 8001.12±1.96 | 3.8 | 101.5 |
| 10000.00 | 10000.07±1.23 | 2.5 | 100.5 |

| Table. 2 - Precision and accuracy (Analysis with spiked samples at three different concentrations) |
| Spiked Plasma Concentration (pg/ml) | Within-run (Intra-day) | Between-run (Inter-Day) |
| Concentration measured (n=6;pg/ml;mean±S.D) | %CV | %Accuracy | Concentration measured (n=6;pg/ml;mean±S.D) | %CV | %Accuracy |
| 15.00 | 14.8±0.07 | 5.6 | 99.8 | 14.9±0.08 | 3.2 | 99.93 |
| 3000.00 | 3002.34±1.23 | 2.1 | 102.4 | 2999.78±2.56 | 1.6 | 99.45 |
| 7000.00 | 6999.4±2.45 | 3.7 | 99.8 | 7004.33±3.61 | 2.4 | 103.45 |

| Table. 3 - Stability studies of Brigatinib in plasma |
| Spiked Plasma concentration (pg/ml) | Room temperature Stability | Processed sample Stability | Long term stability | Freeze and thaw stability |
| Concentration measured (n=6;pg/ml;mean±S.D) | %CV (n=6) | Concentration measured (n=6;pg/ml;mean±S.D) | %CV (n=6) | Concentration measured (n=6;pg/ml;mean±S.D) | %CV (n=6) |
| 15.00 | 14.9±0.12 | 7.8 | 15.5±2.16 | 5.3 | 15.2±1.54 | 8.8 | 14.8±0.12 | 5.8 |
| 7000.00 | 7005.3±0.14 | 8.9 | 7001.3±1.23 | 9.5 | 6999.36±4.012 | 9.4 | 7001.4±2.55 | 2.7 |