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Validation of LC-MS/MS Electrospray Ionisation method for the Estimation of Binimetinib in Human Plasma

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ABSTRACT

A rapid, specific and robust bioanalytical method for quantification of Binimetinib was developed and validated in micro volumes (300 µL) of human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry in positive ion mode. Binimetinib-13C2-D4 was used as an internal standard. Precursor to product ion transitions of *m/z* 442.2 (parent ion) to 363.9 m/z (product ion) and *m/z* 448.5 m/z (parent ion) to 363.9 m/z (product ion) were used to measure the analyte and the internal standard (ISTD), respectively. Chromatographic separation was carried out in reverse phase conditions using XTerra MS C18 Column, 125Å, 3.5 µm, 1 mm X 150 mm with an isocratic mobile phase consisting of Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v) at a flow rate of 0.5 mL min^{-1}. The extraction procedure yielded a recovery of 94.12 and 85.92% for Binimetinib and the internal standard, respectively. The assay exhibited a linear dynamic range of 20.00–200.00pg/mL. The RSD% of intra- and inter-day assay was ≤15%. For its sensitivity, reliability and lower plasma volume requirement, the proposed method is suitable for pharmacokinetic studies.

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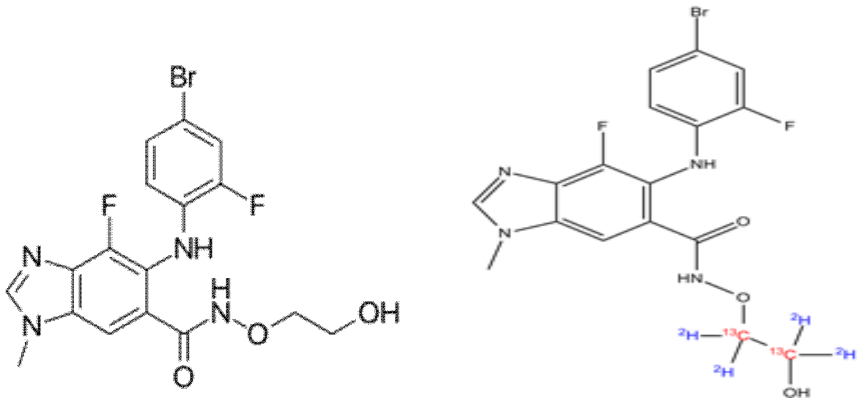
INTRODUCTION

Binimetinib (BT) is an orally available inhibitor of mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) with potential antineoplastic activity. Binimetinib, noncompetitive with ATP, binds to and inhibits the activity of MEK1/2. Inhibition of MEK1/2 prevents the activation of MEK1/2 dependent effector proteins and transcription factors, which may result in the inhibition of growth factor-mediated cell signalling [1,2, 3, 4, 5, 6].

This may eventually lead to an inhibition of tumor cell proliferation and an inhibition in production of various inflammatory cytokines including interleukin-1, -6 and tumor necrosis factor. MEK1/2 are dual-specificity threonine/tyrosine kinases that play key roles in the activation of the RAS/RAF/MEK/ERK pathway and are often up regulated in a variety of tumor cell types [4, 5, 6, 7, 8, 9, 10, 11].

MEK proteins are upstream regulators of the extracellular signal-related kinase (ERK) pathway. In vitro, binimetinib inhibited extracellular signal-related kinase (ERK) phosphorylation in cellfree assays as well as viability and MEK-dependent phosphorylation of BRAF-mutant human melanoma cell lines. Binimetinib also inhibited in vivo ERK phosphorylation and tumor growth in BRAF-mutant murine xenograft models [7, 8, 9, 10, 12, 16].

The chemical name is 5-[(4-bromo-2-fluorophenyl)amino]-4-fluoro-N-(2 hydroxyethoxy)-1-methyl-1H-benzimidazole-6-carboxamide. The molecular formula is C17H15BrF2N4O3 and the molecular weight is 441.2 daltons. Binimetinib is a white to slightly yellow powder. In aqueous media, binimetinib is slightly soluble at pH 1, very slightly soluble at pH 2, and practically insoluble at pH 4.5 and higher. The chemical structure of binimetinib is shown in Figure-1.0 [6, 7, 8, 9].



A **B**

Fig.1: Chemical structures of A) Binimetinib B) Binimetinib-13C2-D4

Literature survey reveals, conventional HPLC methods are not utilizing by the bioanalytical scientists due to limitations in its rapidity, resolution and sensitivity. Hence there is a need for fast or ultra–fast methods such as LC–MS/MS without compromising on the sensitivity and efficiency.

LC–MS/MS methods are widely adopted in bioanalytical applications due to its specificity and high sensitivity. For a bioavailability and bioequivalence studies, it is necessary to quantify the Binimetinib (BT) concentrations in in–vivo samples. Till date, no LC–MS/MS method has been reported for the determination of Binimetinib in any of the biological matrices.

With the above, we made an attempt to develop a specific, sensitive and rapid LC–MS/MS method for simultaneous determination of Binimetinib (BT) in 300 µL of human plasma using Binimetinib-13C-D4 (BTIS) as internal standard and simple Liquid-Liquid extraction method using dichloromethane as extraction solvent shows high-throughput tool for bioanalysis. The developed method was found to be significantly free from the possible matrix interferences and finally validated as per FDA guidelines [18, 19, 20].

MATERIALS AND METHODS

Materials:

Chemical Resources

Binimetinib (BT) was obtained from Arbro Pharmaceuticals, India. Binimetinib-13C2-D4 (BTIS) was procured from Alsachim, France. Water (HPLC Grade), Ammonium acetate (analytical grade) were purchased from Merck, Mumbai, India. Methanol (HPLC Grade) and dichloromethane (HPLC grade) were obtained from J.T. Baker, USA. Human plasma was procured from Navjeevan Blood Blank, Hyderabad. Milli Q water was taken from the in-house Milli-Q system.

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 conditions and Internal standard selection

After a sequence of trials, chromatographic separation was achieved with Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v), gave the best peak shape and low baseline noise was observed using the XTerra MS C18 Column, 125Å, 3.5 µm, 1 mm X 150 mm. The total analysis time was 7 min and flow rate was set to 0.5 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.

For selection of internal standard; Osimertinib and Sunitinib were tried with optimized mobile phase and column conditions. Finally Binimetinib-13C2-D4 (BTIS) was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. The peak elution times for the BT and BTIS were found at 4.49 min and 4.42 ± 0.05min respectively.

Detection

The pure drug (Binimetinib) and Internal standard (Binimetinib-13C2-D4) were prepared in acetontrile (100.00 pg/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 442.2 m/z (parent ion) to 363.9 m/z (product ion) for BT. Similarly, BTIS mass transitions were obtained from 448.5 m/z (parent ion) to 363.9 m/z (product ion). The mass spectrums of parent, product ions were depicted in Figure-2&3.

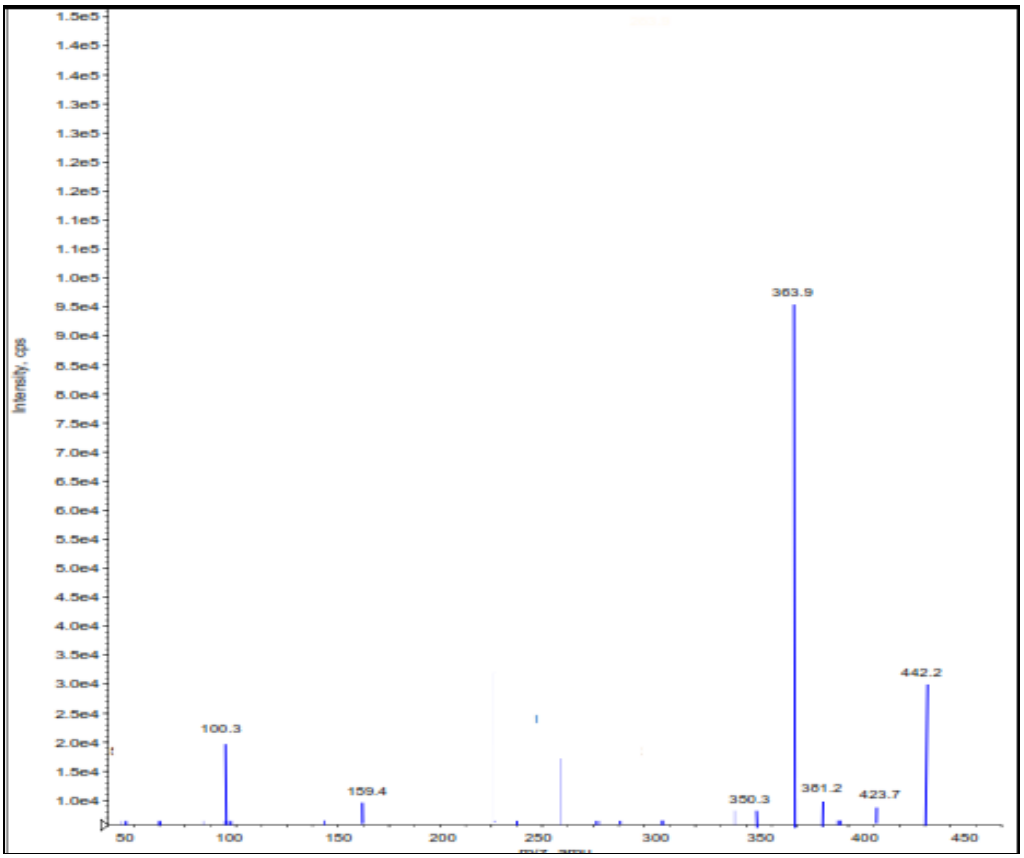


Figure.2: Parent ion mass spectra (Q1) and (Q3) of Binimetinib

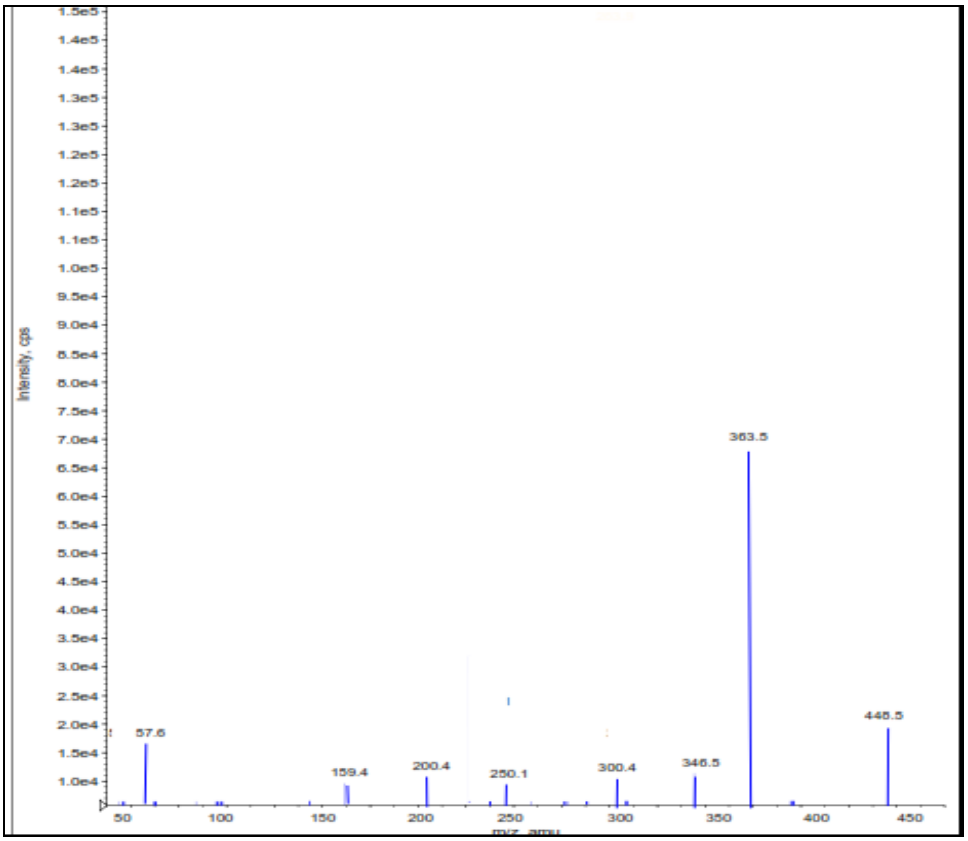


Figure.3: Parent ion mass spectra (Q1) and (Q3) of Binimetinib-13C2-D4

Standard calibration and quality control samples preparation

Standard stock solutions of BT (10.0mg/mL) and BTIS (10.0 mg/mL) were prepared in Methanol. The IS spiking solution (100.0 pg/mL) was prepared in mobile phase solution (Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v)) from BTIS stock solution. Standard stock solutions and IS spiking solutions were stored in refrigerator conditions of 2–8ºC until analysis. Standard stock solutions of LT (10.0 mg/mL) were added to drug-free screened human plasma to obtain concentration levels of 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 pg/mL for analytical standards and 20 (LLOQ), 65 (LQC), 110 (MQC) and 190 pg/mL (HQC) for quality control (QC) standards, and stored in the freezer at -30ºC until analysis. The aqueous standards were prepared in a mobile phase solution (Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v) and stored in the refrigerator at 2–8ºC until analysis.

Sample extraction

The LLE method was used to isolate BT and BTIS from human plasma. For this purpose, 50 µL of BTIS (10 pg/mL) and 300 µL of plasma sample were added to the labelled polypropylene tubes and vortexed briefly for about 10 min. Thereafter, 3mL of extraction solvent of

dichloromethane was added and vortexed for about 10 min. Next, the samples were centrifuged at 6000 rpm for approximately 10 min at ambient temperature. From each, a supernatant sample was transferred into labelled polypropylene tubes and evaporated to a dryness of 55ºC briefly, and then reconstituted with a mobile phase solution (Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v), and the sample was transferred into autosampler vials and injected into the LC-MS/MS for study.

Method validation

The developed method was validated over a linear concentration range of 20.0–200.0 pg/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.

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 drug (Binimetinib) retention time and less than 5% for Internal standard (Binimetinib-13C2-D4) retention time.

Limit of Quantification (LOQ)

Six LLOQ standards were prepared in screened plasma lot along with IS (100.00 pg/ml) and signal to noise ratio (S/N) was calculated using analyst software.

Linearity

Calibration standards were prepared to obtain linearity range of 20, 40, 60, 80, 100, 140, 180 and 200 pg/mL 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 (20.00 pg/ml), Low QC (65.00 pg/ml), Mid QC (110.00 pg/ml) and High QC (190.00 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 (110.00 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 QC (65.00 pg/ml), Mid QC (110.00 pg/ml) and High QC (190.00 pg/ml).

Stability studies

Bench top Stability (Room Temperature Stability, 24 h)

Six replicates of spiked low and high concentrations (BT 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, 55 h)

Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 55 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-30°C, 40 Days)

After completion of the stability period stored at -30 °C (40 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

RESULTS AND DISCUSSION

Method development

On the way to develop a simple and easy applicable method for determination of Binimetinib 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.

After a series of trials a mobile phase consisting of 10mM ammonium acetate and methanol: acetonitrile in varying combinations were tried. Using a mobile phase containing Methanol: 20mM Ammonium acetate (pH: 4.5, Adjusted with diluted aceticacid) (90: 10, v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the XTerra MS C18 Column, 125Å, 3.5 µm, 1 mm X 150 mm analytical column with a flow rate of 0.5 ml/min and reduced runtime to 7 min. The column oven temperature was kept at a constant temperature of about 38 °C and temperature of auto sampler was maintained at 4°C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography.

Binimetinib-13C2-D4 (BTIS) was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions in terms of better extractability.

The peak elution times for the Binimetinib and Binimetinib-13C2-D4 were found at 4.49 min and 4.42 ± 0.05min, respectively with runtime 7 min. Various organic solvents and buffers were optimized to extract BT and BTIS from the plasma sample. After a series of trials, dichloromethane was selected as appropriate due to high recovery efficiency and matrix free interference.

The predominant peaks in the primary ESI spectra of Binimetinib and Binimetinib-13C2-D4 were obtained using MRM positive ion mode with mass transitions of 442.2 m/z (parent ion) to 363.9 m/z (product ion) and 448.5 m/z (parent ion) to 363.9 m/z (product ion). respectively.

Method validation

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed at retention times of Binimetinib and Binimetinib-13C2-D4 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 20.0 pg/ml. Represent chromatograms were shown in Figure 4-6.

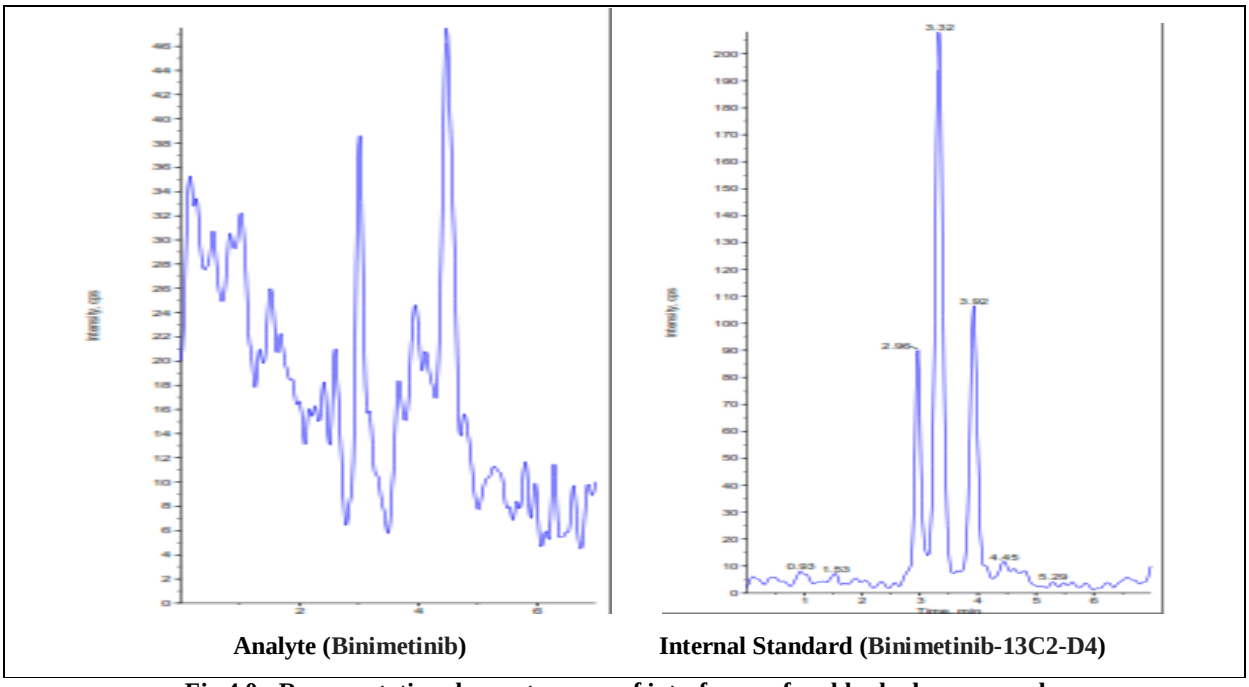


Fig.4.0 - Representative chromatograms of interference free blank plasma sample

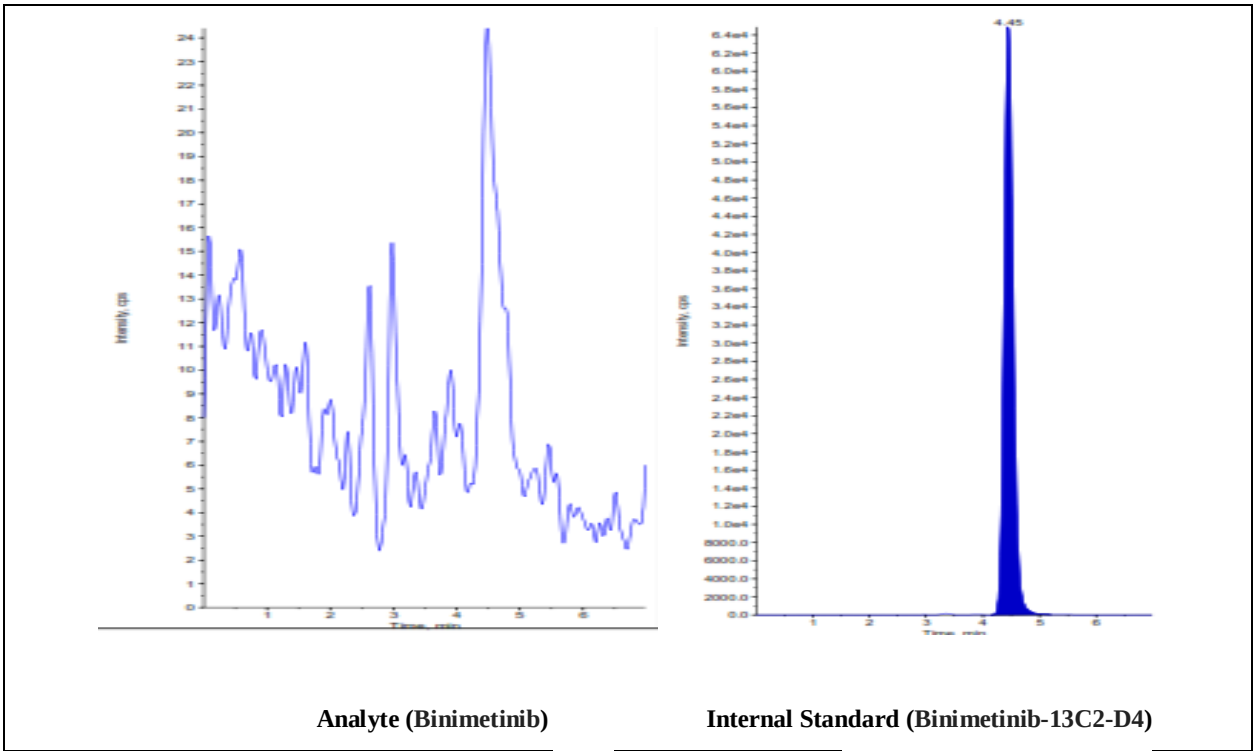
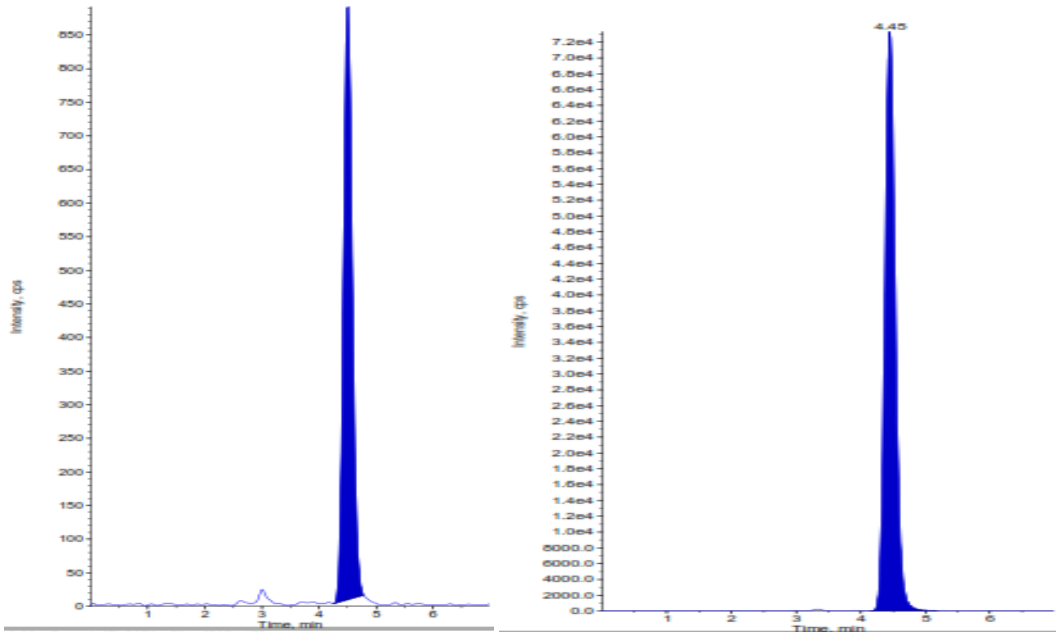


Fig. 5 - Representative chromatograms of blank plasma samples and Binimetinib-13C2-D4 (BTIS)



Analyte (Binimetinib) **Internal Standard (Binimetinib-13C2-D4)**

Fig. 6- Chromatogram of LLOQ sample (Binimetinib and Binimetinib-13C2-D4)

Linearity

Linearity was plotted as a peak area ratio (Binimetinib peak area / Binimetinib-13C2-D4 peak area) on the y-axis against Talazoparib concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for Binimetinib over a linearity range of 20 to 200.00 pg/ml. The correlation coefficient was greater than 0.9990 for Talazoparib. The %CV was less than 15% and mean %accuracy was ranged between 99.63 – 101.45%. Results were presented in Table 1.

Table. 1 - Calibration curve details of Binimetinib

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
20.00	20.13±0.56	2.78	100.63
40.00	40.38±0.66	1.62	100.96
60.00	59.78±0.33	0.56	99.63
80.00	80.79±1.04	1.29	100.98
100.00	100.38±0.84	0.83	100.38
140.00	142.03±1.03	0.72	101.45
180.00	179.67±1.67	0.93	99.81
200.00	201.20±0.71	0.35	100.60

Precision & Accuracy

Intra and inter batch %accuracy for Binimetinib was ranged between 98.41-100.14 and 96.93-104.39. %CV is 0.25-4.75 and 1.32-3.23. Results are presented in Table 2.

Table.2-Precision and accuracy (Analysis with spiked samples at three different concentrations) of Binimetinib

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
65.0	63.97±2.17	3.40	98.41	63.00±2.04	3.23	99.93
110.00	109.76±0.45	0.41	99.78	110.36±1.75	1.58	100.33
190.00	190.27±0.47	0.25	100.14	191.83±2.54	1.32	100.96

Recovery

The mean %recovery for LQC, MQC, HQC samples of Binimetinib were 97.64%, 97.30% and 87.42% respectively.

The overall mean %recovery and %CV of Binimetinib across QC levels is 94.12% and 2.30%. For the Binimetinib-13C2-D4 (internal standard) the mean % recovery and %CV is 85.92% and 4.82%.

Matrix Effect

No significant matrix effect found in different sources of rat plasma tested for Binimetinib, Binimetinib-13C2-D4. The %CV was found to be 7.72.

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

Quantification of the Binimetinib 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.

Table. 3 - Stability studies of Binimetinib in spiked plasma samples

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	24h		55h		40 days		Cycle (48h)	
	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)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)
65.00	65.54±4.21	6.74	62.33±1.80	2.89	63.20±2.05	3.24	64.69±3.27	5.06
190.0	192.03±0.51	0.26	191.18±1.46	0.76	191.05±2.81	1.47	191.67±1.74	0.91

CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 20–200.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 15% and %accuracy ranged from 96.93-104.39%. The overall %recovery for Binimetinib, Binimetinib-13C2-D4 was greater than 85%. 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 liquid-liquid extraction with run time of 7.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Binimetinib.

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CONFLICT OF INTEREST: Authors declare that, there is no conflict of interest.

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