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Advances in Bioresearch

## ORIGINAL ARTICLE

# Development of RP-UPLC Method for the Simultaneous Quantification of Binimetinib and Encorafenib in Dosage Form; Application to Stability Study

**Kolli Lavanya[1], Dasireddy Saisri[1], Pedada Nirosha[1], R.A.V.N. Sai Charan D[2], G. Divya [1*]**
[1]Sri Venkateswara college of pharmacy, Etcherla, 532410. Under the department of pharmaceutical analysis, Andhra University, Visakhapatnam.
[2]Ctruecure Biotech LLP, Hyderabad, Telangana.
*Corresponding author email: divyagolivi07@gmail.com (ORCID: 0009-0004-8474-2374)

**ABSTRACT**
*A simple, accurate and precise method was developed for the simultaneous estimation of the BMTB and ECRB in Tablet dosage form by RP-UPLC technique. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min respectively. Chromatographic elution was processed through the mobile phase composition of 0.01N KH2PO4 buffer (3.5 pH) and acetonitrile in the ratio of 60:40%v/v pumped through an Zorbax C18 Column (100 x 3 mm, 2.1 m) reverse phase column, at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 294 nm. Based on the solubility, all the dilutions were made with acetonitrile and water in the ratio of 60:40%v/v. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min respectively. An injection volume of 0.30 ml was infused through an UPLC system to get the better performance. Repeatability of the method was determined in the form of %RSD and findings were 0.6 and 0.3 for BMTB and ECRB respectively. LOD, LOQ values obtained from regression equations of ECRB and BMTB were 0.51, 1.55µg/ml and 1.47, 4.44 µg/ml respectively. Two analytes were subjected for acid, peroxide, photolytic, alkali, neutral and thermal degradation studies and the results shown that the percentage of degradation was found between 0.76% and 6.88%. Retention times and total run time of two drugs were decreased and the developed method was simple and economical. So, the developed method can be adopted in industries as a regular quality control test for the quantification of BMTB and ECRB.*
**Keywords:** Binimetinib, Encorafenib, Stability studies, UPLC, Validation.

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## INTRODUCTION

The ECRB is a drug component available in the market as Braftovi, utilized in the treatment of different melanomas. It belongs to a BRAF inhibitor that affects the key enzymes involved in the mitogen-activated protein kinase (MAPK) signaling path. This path of signaling takes place in many kinds of cancers, together with colorectal cancer and melanomas.[1,2] ECRB inhibits the ATP-competitive RAF kinase, downregulates cyclin-D1, and decreases ERK phosphorylation.[3-5] This stops the cell cycling process in phase-G1, prompting senescence without apoptosis. ECRB chemically designated as methyl[(2S)-1-{[4-(3-{5-chloro- 2-fluoro-3-[(methylsulfonyl) amino] phenyl}-1-isopropyl- 1H-pyrazol-4-yl)-2-pyrimidinyl] amino}-2-propanyl] carbamate[1,3] with molecular weight and formula of 540.011 g/mole and $C_{22}H_{27}ClFN_7O_4S$, respectively (Fig. 1a). BMTB (trade name Mektovi) selectively inhibits MEK, a central kinase in the tumor stimulating MAPK-path. Incongruous stimulation of the path has been shown to ensue in several cancers.[6] BMTB is a mitogen-activated protein kinase (MEK) inhibitor available orally, or, more specifically, an inhibitor of MAP2K.[7] MEK is part of the rat sarcoma (RAS) pathway, which is involved in cell proliferation and survival. MEK is upregulated in many forms of cancer.[8] BMTB, uncompetitive with ATP, binds to and inhibits the activity of MEK1/2 kinase, which has been shown to

regulate several key cellular activities, including proliferation, survival, and angiogenesis. BMTB chemically designated as 5-((4-bromo-2-fluorophenyl) amino)-4-fluoro- N-(2-hydroxyethoxy)-1-methyl-1H-benzo[d] imidazole- 6-carboxamide[6-8] with molecular weight and formula of 441.23 g/mole and $C_{17}H_{15}BrF_2N_4O_3$, respectively (Fig. 1b).

ECRB and BMTB target two dissimilar kinases in the path of RAS-RAF-MEK-ERK. A combination of ECRB and BMTB results in superior anti-proliferating action *in vitro* in BRAF mutation-positive cell lines, compared with activity of any single drug alone.[7] In addition to the above, these two drug combinations acted to defer the emergence of resistance in BRAF-V600E mutant human melanoma xenografts in mice compared with the administration of any one drug alone.[4,8]

The literature review unveils that very less UPLC-MS/ MS[9] and reverse-phase high performance liquid chromatographic (RP-HPLC)[10] techniques have been established for the determination of ECRB and BMTB. Based on the reported HPLC methods, there is a need to develop a stability-indicating RP-UPLC method for the simultaneous estimation of ECRB and BMTB in bulk and dosage form.

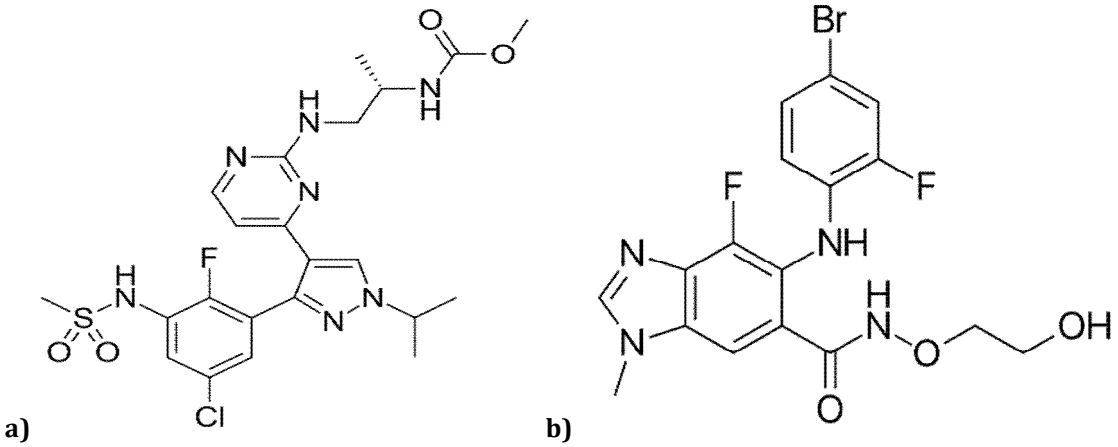


**Fig. 1: Chemical structures of a) Encorafenib; b) Binimetinib.**

## MATERIAL AND METHODS

### Chemicals and Reagents

API of ECRB and BMTB were obtained from spectrum Pharma Research Solutions, Hyderabad. HPLC-grade methanol and acetonitrile were procured from Merck chemical division, Mumbai, India, Potassium dihydrogen ortho phosphate, orthophosphoric acid, sodium dihyrogen ortho phosphate and HPLC-grade water were bought from Rankem, avantor performance material india limited. Braftovi capsules and Mektovi tablets were obtained from local pharmacy.

### Method development

During the method development various mobile phase compositions consisting of methanol, acetonitrile, water, phosphate buffers and different stationary phases were executed to get fine chromatographic conditions like theoretical plates, resolution, tailing and peak shape. The processed trials were mentioned below:

### Optimized conditions

Liquid chromatographic UPLC system of Waters equipped with PDA (photodiode array detector), auto-sampling unit Zorbax C18 Column (100 x 3 mm, 2.1 µ) reverse phase column. The mobile phase composition of 0.01N $KH_2PO_4$ buffer (3.5 pH) and acetonitrile in the ratio of 60:40 was pumped through a column at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 294 nm. Integration of output signals were monitored and processed by waters Empower software-2.0.

### Diluent

Depending up on the solubility of the drugs, diluent was optimized. Initially dissolved in methanol and diluted with acetonitrile and water (50:50).

### Preparation of Standard Stock Solutions

Exactly weighed 90mg of ECRB and 9mg of BMTB poured in to two 50ml volumetric flasks alone. 10ml of diluent was added and vortexed for 20 min. Flasks were made up with water and acetinitrile (50:50) and marked as standard stock solution 1and 2 (1800µg/ml of ECRB and 180µg/ml BMTB). 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent to get 180µg/ml of ECRB and 18µg/ml of BMTB.

**Preparation of Sample Stock Solutions**

5 tablets were weighed and the average weight of each tablet was calculated. The weight equivalent to 1 tablet was transferred into a 500ml volumetric flask and 25 ml of diluent was added and sonicated for 25 min. Further the volume was made up with diluent and filtered through 0.45 µ filter (900µg/ml of ECRB and 90 µg/ml of BMTB). 2ml of the resultant solution was poured in to a 10ml volumetric flask and made up with diluent (180µg/ml of ECRB and 18µg/ml of BMTB).

**Preparation of Buffer**

Accurately weighed 1.36 gm of potassium dihyrogen orthophosphate in a 1000 ml of volumetric flask and add about 900 ml of milli-Q water. Sonicate the solution for 10 min, make up the volume with water and then adjust the pH to 3.5 with 0.1% orthophosphoric acid solution**.**

**Method Validation**

The developed method for ECRB and BMTB was subjected for validation for the parameters like system suitability, linearity, robustness, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy as per the guidelines of ICH[11, 12].

**RESULTS AND DISCUSSION**

Finally, excellent chromatographic efficiency parameters were obtained with the mobile phase composition of 0.01N $KH_2PO_4$ buffer (3.5 pH) and acetonitrile in the ratio of 60:40%v/v pumped through an Zorbax C18 Column (100 x 3 mm, 2.1 µ) reverse phase column, at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 294 nm. Based on the solubility, all the dilutions were made with acetonitrile and water in the ratio of 60:40%v/v. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min respectively (Fig. 2). An injection volume of 0.30 µl was infused through an UPLC system to get the better performance.

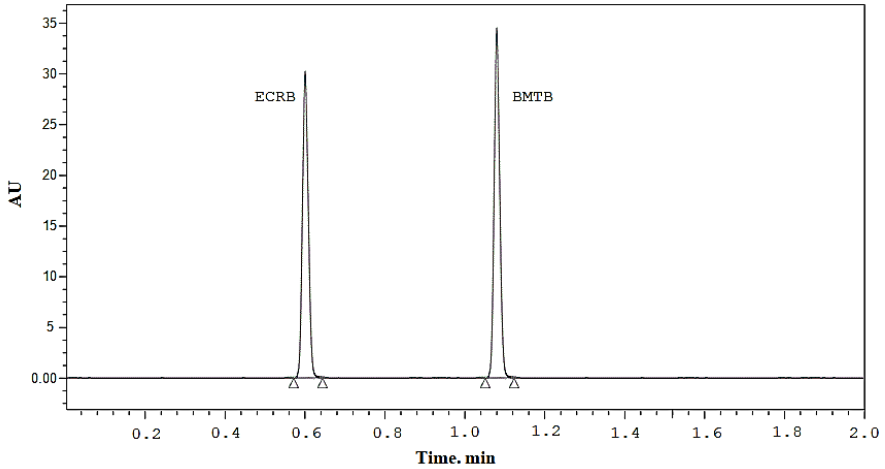


**Fig. 2: Optimized Chromatogram of ECRB and BMTB**

**Method validation**

**System Suitability**

The system suitability variables were estimated by preparing standard solutions of ECRB and BMTB and the same were injected 6 times in to the chromatographic system. The variables like peak tailing, resolution and USP plate count were estimated[12-18]. The results were shown in Fig. 3 and Table 1.

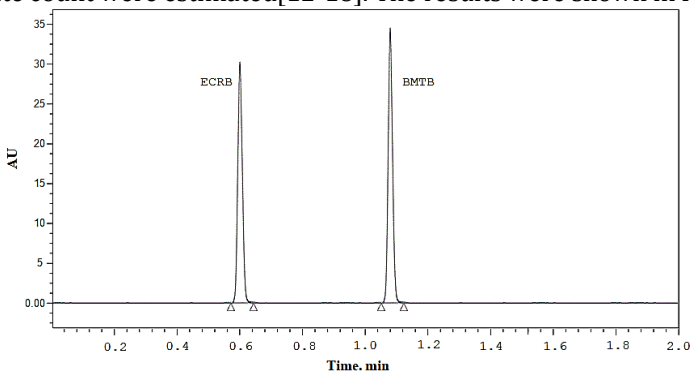


**Fig. 3: Chromatogram showing standard injection.**

**Table 1: System suitability parameters for ECRB and BMTB**

| S.No | ECRB | | | BMTB | | | |
|---|---|---|---|---|---|---|---|
| | RT(min) | USP Plate Count | Tailing | RT(min) | USP Plate Count | Tailing | USP Resolution |
| 1 | 0.766 | 2732 | 1.22 | 1.128 | 3728 | 1.16 | 5.2 |
| 2 | 0.766 | 2641 | 1.27 | 1.128 | 3585 | 1.19 | 5.0 |
| 3 | 0.766 | 2624 | 1.26 | 1.129 | 3583 | 1.19 | 5.0 |
| 4 | 0.767 | 2625 | 1.27 | 1.130 | 3625 | 1.18 | 5.0 |
| 5 | 0.767 | 2686 | 1.24 | 1.130 | 3548 | 1.18 | 4.9 |
| 6 | 0.767 | 2599 | 1.27 | 1.130 | 3453 | 1.19 | 4.8 |

## Specificity

Method specificity was determined by infusing the blank, placebo, standard and sample solutions in to a chromatographic system and the resulting chromatograms were evaluated for interference with the excipients, degradants and other components may expected to be present. Blank, standard, formulation and placebo chromatograms were represented in Fig. 4.

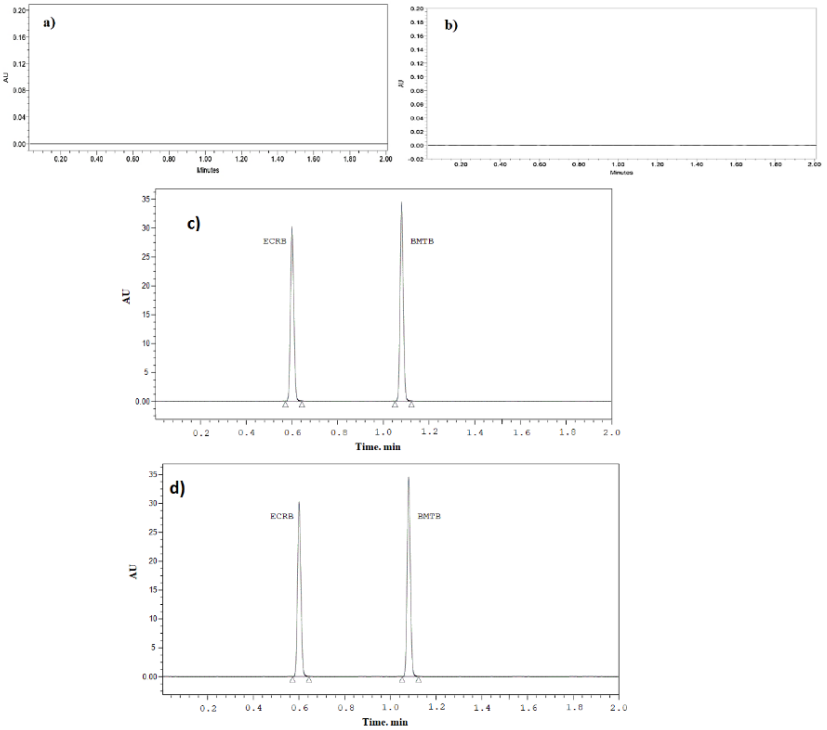


**Fig. 4: Chromatograms of a) Blank, b) Placebo, c) Standard and d) Sample.**

## Precision

Precision of the method was evaluated in terms of method precision and intermediate precision. The method precision (repeatability) was estimated by infusing 6 standard solutions and 6 sample solutions. Intermediate precision was evaluated by infusing 6 standard solutions and 6 sample solutions on different days by different employees on different chromatographic systems [16]. The peak responses of all the chromatograms were taken and standard deviation, % RSD (relative standard deviation) and percentage assay of sample solutions were calculated. The findings were represented in Tables 2 and 3.

**Table 2: Repeatability results of ECRB and BMTB.**

| S. No | Area of ECRB | Area of BMTB |
|---|---|---|
| 1. | 1246477 | 243254 |
| 2. | 1252281 | 243393 |
| 3. | 1251131 | 242062 |
| 4. | 1248204 | 245139 |
| 5. | 1242873 | 242591 |
| 6. | 1252694 | 245621 |
| Mean | 1248943 | 243677 |
| SD | 3833.3 | 1411.7 |
| %RSD | 0.3 | 0.6 |

SD: standard deviation; RSD: relative standard deviation.

**Table 3: Intermediate precision results of ECRB and BMTB.**

| S. No | Area of ECRB | Area of BMTB |
|---|---|---|
| 1. | 1246469 | 243850 |
| 2. | 1244094 | 242664 |
| 3. | 1243469 | 245656 |
| 4. | 1237521 | 242089 |
| 5. | 1233073 | 245904 |
| 6. | 1236125 | 243739 |
| Mean | 1240125 | 243984 |
| SD | 5285.7 | 1542.3 |
| %RSD | 0.4 | 0.6 |

SD: standard deviation; RSD: relative standard deviation.

**Accuracy**

Method accuracy was estimated at three variable concentrations of 50%, 100%, and 150% level by spiking the known amount of the drug analytes. The % recovery at each level was calculated and the findings were represented in Table 4 (Fig. 5 to 7).

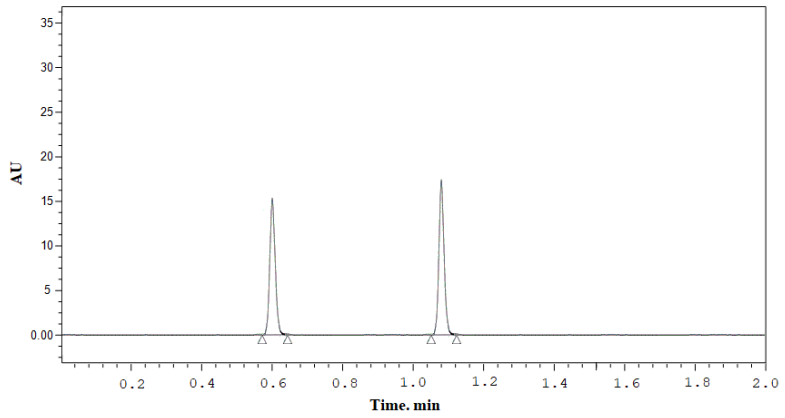


**Fig.5: Chromatogram showing accuracy 50%injection**

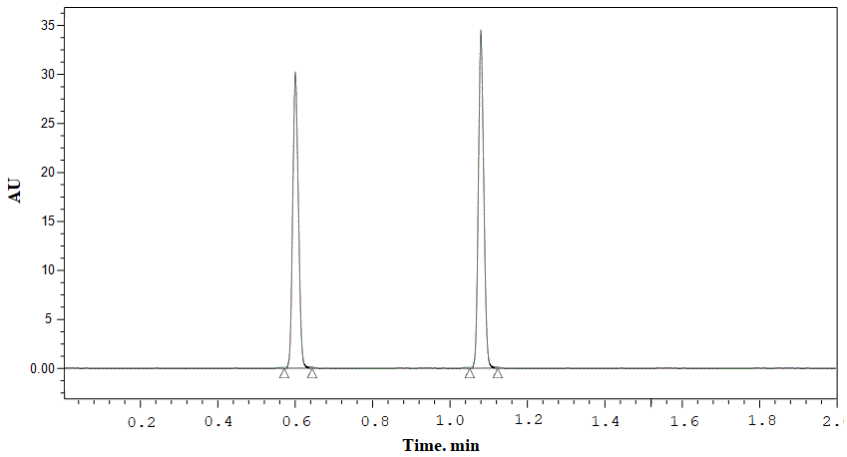


**Fig 6:Chromatogram showing accuracy 100%injection**

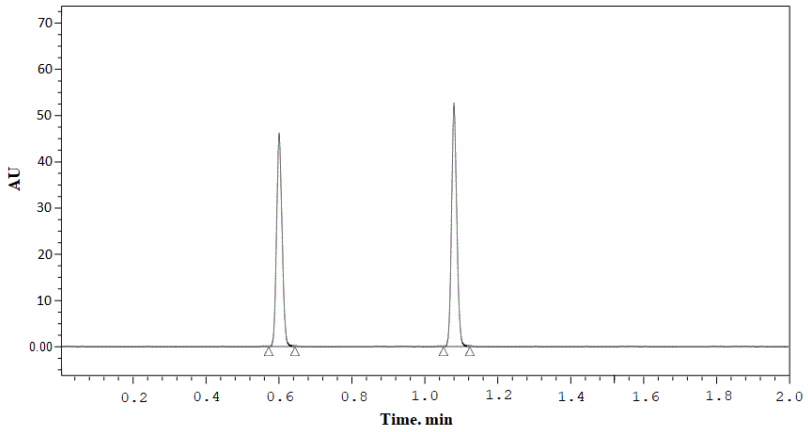


**Fig 7: Chromatogram showing accuracy 150%injection.**

**Table 4: Accuracy results of ECRB and BMTB.**

| % Level | Amount Spiked (µg/ml) | Amount recovered (µg/ml) | % Recovery | Mean % Recovery | Amount Spiked (µg/ml) | Amount recovered (µg/ml) | % Recovery | Mean %Recovery |
|---|---|---|---|---|---|---|---|---|
| 50% | 90 | 89.72457 | 99.69 | 99.59% | 9 | 8.990776 | 99.90 | 99.70% |
| | 90 | 89.71574 | 99.68 | | 9 | 8.988794 | 99.88 | |
| | 90 | 89.68253 | 99.65 | | 9 | 8.993215 | 99.92 | |
| 100% | 180 | 179.4969 | 99.72 | | 18 | 17.8281 | 99.04 | |
| | 180 | 179.2611 | 99.59 | | 18 | 17.91439 | 99.52 | |
| | 100 | 99.08887 | 99.09 | | 18 | 17.93818 | 99.66 | |
| 150% | 270 | 269.1872 | 99.70 | | 27 | 26.95213 | 99.82 | |
| | 270 | 268.6239 | 99.49 | | 27 | 26.97965 | 99.92 | |
| | 270 | 269.2932 | 99.74 | | 27 | 26.88817 | 99.59 | |

**Linearity**

Linearity of the developed method was evaluated by processing 6 different concentration levels of both ECRB and BMTB over the concentration of 45 to 270 µg/ml and 4.5 to 27 µg/ml. Each concentration level was processed in triplicate[17]. The linearity plots were acquired by plotting peak response (on X-axis) versus concentration (on Y-axis). The results of the linearity were represented in Fig. 8, 9 and Table 5.

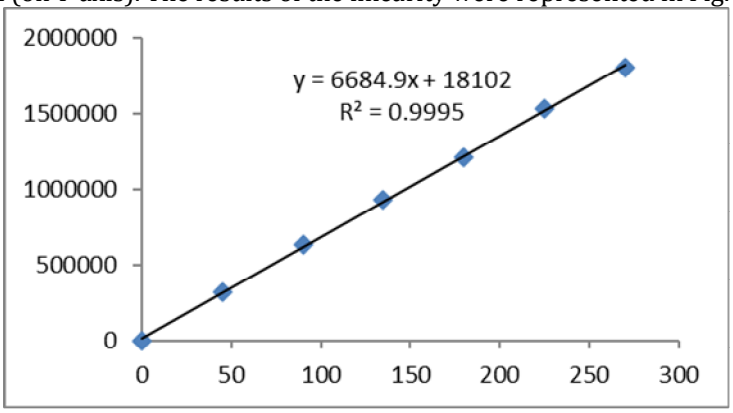


**Fig. 8: Calibration curve of Encorafenib**

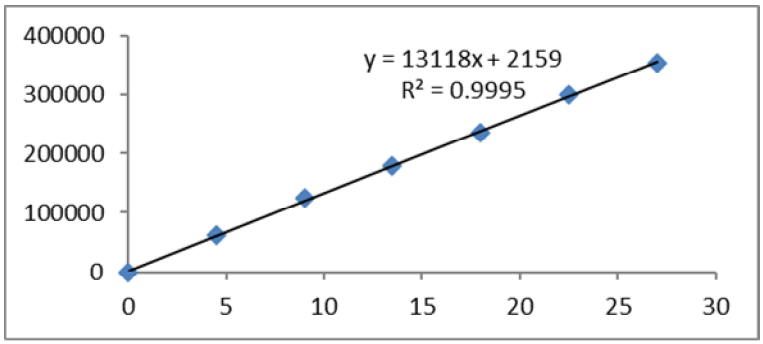


**Fig. 9: Calibration curve of Binimetinib.**

**Table 5: Linearity results for ECRB and BMTB.**

| ECRB | | BMTB | |
|---|---|---|---|
| **Conc (µg/ml)** | **Peak area** | **Conc (µg/ml)** | **Peak area** |
| 0 | 0 | 0 | 0 |
| 45 | 322448 | 4.5 | 60978 |
| 90 | 633298 | 9 | 123832 |
| 135 | 934969 | 13.5 | 179592 |
| 180 | 1211063 | 18 | 235754 |
| 225 | 1536452 | 22.5 | 300887 |
| 270 | 1805748 | 27 | 353700 |

**LOD and LOQ**

LOD is lowest quantity of drug in a sample that can be identified but cannot be quantify exactly. LOQ is the lowest quantity of a drug in an analyte which can be quantitatively estimated with a suitable accuracy and precision. The LOD and LOQ values were calculated from the linearity data by utilizing standard deviation and slope of the curve. The resulting LOD and LOQ findings were represented in Table 6.

**Table 6: LOD and LOQ results for ECRB and BMTB.**

| Analyte | LOD (µg/ml) | LOQ (µg/ml) |
|---|---|---|
| ECRB | 0.51 | 1.55 |
| BMTB | 1.47 | 4.44 |

**Robustness**

The method robustness was processed by introducing small variation in the optimized LC conditions such as organic phase in mobile phase (±5%), flow rate (-0.27 and +0.33 ml/ min) and column temperature (±5°C). The findings were shown in the Table 7.

**Table 7: Robustness data for ECRB and BMTB**

| S.No. | Variation in LC conditions | ECRB % RSD | BMTB % RSD |
|---|---|---|---|
| 1 | Flow rate (-) 0.27ml/min | 0.8 | 0.7 |
| 2 | Flow rate (+) 0.33ml/min | 0.5 | 0.5 |
| 3 | Organic phase -5% | 0.6 | 0.3 |
| 4 | Organic phase + 5% | 0.6 | 0.6 |
| 5 | Temperature at 25°C | 1.1 | 0.9 |
| 6 | Temperature at 35°C | 1.0 | 0.8 |

**Degradation Studies**

**Alkali Degradation Studies**

To 1 ml of each stock solution of ECRB and BMTB, 1 ml of 2N NaOH was added in to a 10 ml volumetric flask and kept at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes [12]. The findings were represented in Table 8 and Fig. 10.

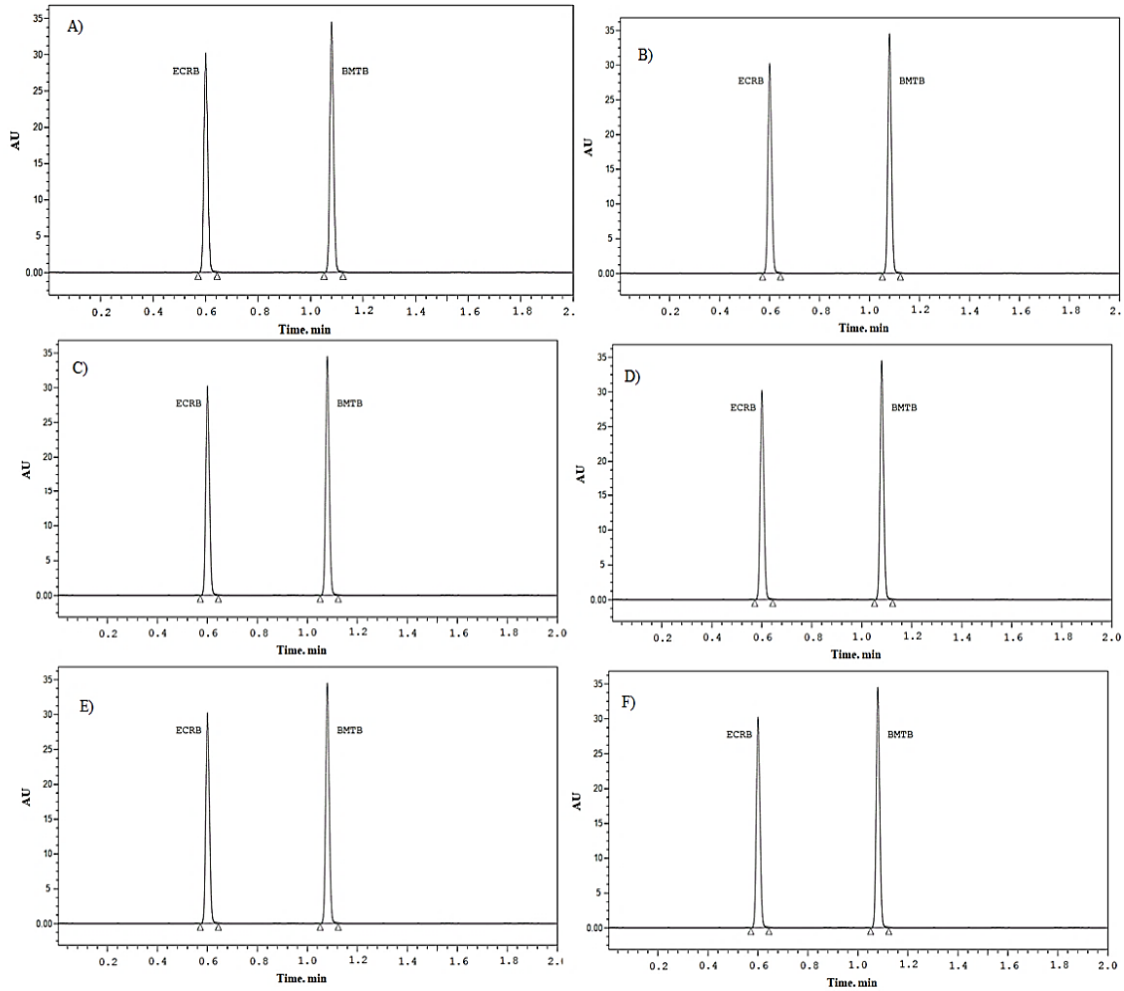


**Fig. 10: Chromatograms for A) alkali, B) photo, C) acid, D) neutral, E) oxidation, and F) dry heat degradation study.**

**Table 8: Degradation data of ECRB and BMTB.**

| Type of degradation | ECRB | | | BMTB | | |
|---|---|---|---|---|---|---|
| | Area | %Recovered | % Degraded | Area | %Recovered | % Degraded |
| Acid | 1211851 | 97.47 | 2.53 | 237330 | 97.34 | 2.66 |
| Alkali | 1202123 | 96.69 | 3.31 | 236412 | 96.97 | 3.03 |
| Peroxide | 1157758 | 93.12 | 6.88 | 231328 | 94.88 | 5.12 |
| Thermal | 1218460 | 98.00 | 2.00 | 236704 | 97.08 | 2.92 |
| UV light | 1208181 | 97.18 | 2.82 | 239749 | 98.33 | 1.67 |
| Neutral | 1225794 | 98.59 | 1.41 | 241947 | 99.24 | 0.76 |

**Photolytic Stability Study**

For the photolytic stability study, ECRB 1800µg/ml and BMTB 180µg/ml solutions were exposed to UV-light by placing the solutions in UV cabinet for 1day or 200 Watt hours/$m^2$ in photo stability chamber. The resulting solutions were combined in a 10 volumetric flask and made up to the mark with diluent to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

**Acid Degradation Studies**

To 1 ml of each stock solution of ECRB and BMTB, 1 ml of 2N Hydrochloric acid was added in to a 10 ml volumetric flask and refluxed at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

**Neutral Degradation Studies**

To 1 ml of each stock solution of ECRB and BMTB, 5 ml of water was added in to a 10 ml volumetric flask and kept for refluxing at 60°C for 1 h. Further, the resulting solution was made up to the mark to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

**Oxidation**

To 1 ml of each stock solution of ECRB and BMTB, 1 ml of 20% hydrogen peroxide (H2O2) were added in to a 10 ml volumetric flask and kept at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

**Dry Heat Degradation Studies**

To a 10 ml volumetric flask add 1ml each stock solution of ECRB and BMTB and monitored at 105°C for 1 h in an hot air oven to perform the dry heat stability study [18]. Further, the resulting solution was made up to the mark to get 180µg/ml and 18µg/ml concentrations of ECRB and BMTB respectively. From that 0.30 µl of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

After the method development trials, chromatographic parameters were optimized with the mobile phase composition of 0.01N KH2PO4 buffer (3.5 pH) and acetonitrile in the ratio of 60:40%v/v pumped through an Zorbax C18 Column (100 x 3 mm, 2.1 m) reverse phase column, at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 294 nm. Based on the solubility, all the dilutions were made with acetonitrile and water in the ratio of 60:40%v/v. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min respectively. An injection volume of 0.30 ml was infused through an UPLC system to get the better performance. Optimized method was subjected for the validation as per the ICH guidelines. In the system suitability studies, plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min in that order. We did not found any additional peaks in blank and placebo at retention times of these drugs in this technique. So this technique was said to be specific.

Average area, SD and % RSD were calculated for the method and intermediate precision and the %RSD values were less than 0.6% for ECRB and BMTB. As the limit of precision was < 2 and both the precisions were passed in this analysis process. The method has high degree of accuracy based on the mean recovery values and were found to be 99.59% and 99.70% for ECRB and BMTB respectively. The correlation coefficient values obtained for both the drugs were >0.999 and it proves that the method has high degree of linearity. The method robustness was processed by variation in mobile phase, flow rate and column temperature and % RSD was calculated. The resultant findings (Table 7) prove the method robustness. Further, two analytes were subjected for acid, peroxide, photolytic, alkali, neutral and thermal degradation studies and the results shown that the drugs were prone to degradation between 0.76% and 6.88%.

## CONCLUSION

A simple, accurate and precise method was developed for the simultaneous estimation of the ECRB and BMTB in Tablet dosage form by RP-UPLC technique. Retention times of ECRB and BMTB were found to be 0.613 min and 1.086 min respectively. Chromatographic elution was processed through the mobile phase composition of 0.01N KH2PO4 buffer (3.5 pH) and acetonitrile in the ratio of 60:40%v/v pumped through an Zorbax C18 Column (100 x 3 mm, 2.1 m) reverse phase column, at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 294 nm. Based on the solubility, all the dilutions were made with acetonitrile and water in the ratio of 60:40%v/v. Repeatability of the method was determined in the form of %RSD and findings were 0.3 and 0.6 for ECRB and BMTB respectively. LOD, LOQ values obtained from regression equations of ECRB and BMTB were 0.51, 1.55µg/ml and 1.47, 4.44 µg/ml respectively. Two analytes were subjected for acid, peroxide, photolytic, alkali, neutral and thermal degradation studies and the results shown that the percentage of degradation was found between 0.76% and 6.88%.

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