RESEARCH ARTICLE

Development of an RP-HPLC Method for the Simultaneous Quantification of Encorafenib and Cetuximab in their Bulk Form

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ABSTRACT

The primary goal of this investigation was to develop and validate a high-performance liquid chromatograophy (HPLC) method that is straightforward, reliable, sensitive, and accurate in quantifying the concentrations of encorafenib and cetuximab in bulk form. A Waters HPLC system with an autosampler and a PDA detector was used to create the HPLC technique. With a particle size of 5 µ and dimensions of 250 x 4.6 mm, a Waters X-Terra RP-18 column was used for the separation. A combination of acetonitrile and 0.1% triethylamine (TEA) at a pH of 2.5 makes up the mobile phase. Acetonitrile to TEA is 40:60 in ratio. A 240 nm wavelength, a 10 µL injection volume, and a flow rate of 1.0 mL/min were used for the chromatographic procedure. Analyte serial dilutions will be evaluated in order to establish linearity. The stock solutions with concentrations ranging from 1.25 to 7.50 µg/mL for cetuximab and 18.75 to 112.50 µg/mL for encorafenib were used to generate these dilutions. The dilutions were made by adding the mobile phase to the stock solutions to create a series. Encorafenib and cetuximab were found to have limit of detection (LoD) and limit of quantitation (LoQ) values of 0.225 and 0.015 g/mL and 0.75 and 0.05 g/mL, respectively. The method that was developed showed a high level of accuracy, as evidenced by the relative standard deviation (RSD) being less than 2%. The developed analytical method for estimating encorafenib and cetuximab is both straightforward and accurate.

Keywords: Encorafenib, Cetuximab, HPLC, PDA detector, Wavelength.

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INTRODUCTION

Braftovi, also known as encorafenib, is a pharmaceutical compound that is given to treat specific subtypes of melanoma cancer. This is a small drug that specifically inhibits important enzymes in the MAPK signaling pathway by targeting BRAF. This route is seen in several cancers, including colorectal and melanoma. Novartis first initiated the development of the chemical, which Array BioPharma later took up.[1,2] Adverse responses such as tiredness, nausea, diarrhea, vomiting, stomach discomfort, and arthralgia are experienced by at least 25% of the patients.[3,4] A test allowed by the food and drug administration (FDA) can find the BRAF V600E gene, which is a sign of advanced non-small cell lung cancer. In October 2023, the US FDA gave its approval to this treatment.[5]

Cetuximab is a synthetic hybrid antibody that specifically binds to the epidermal growth factor receptor and efficiently blocks epidermal growth factor attachment. epidermal growth factor receptor (EGFR), a constituent of the ErbB receptor tyrosine kinase family, is present in both healthy and cancerous cells. Its primary function is to control the growth and stability of epithelial tissue. EGFR is frequently overexpressed in malignant cells, which has been associated with more advanced illness and a worse prognosis, making it a significant factor in numerous forms of cancer. EGFR frequently undergoes mutations in specific cancer types and functions as a catalyst for the development of tumors. When evaluated *in-vitro*, cetuximab demonstrated anti-tumor activities in numerous cancer cell lines and human tumor xenografts.[6-8]

The FDA authorized cetuximab under the trade name ERBITUX in February 2004.[9] It is used to treat colorectal cancer that has spread, head and neck cancer, and colorectal cancer that has spread and the BRAF V600E gene. Advanced colon cancer, non-small cell lung cancer expressing EGFR, and unresectable squamous cell skin cancer have also been investigated.[10] This drug, cetuximab, is given through an IV and can be used alone or with other chemotherapies like radiation treatment, platinum drugs, leucovorin, fluorouracil, and irinotecan.[11-13]

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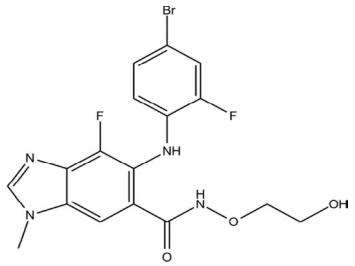

Figure 1: Molecular structure of encorafenib

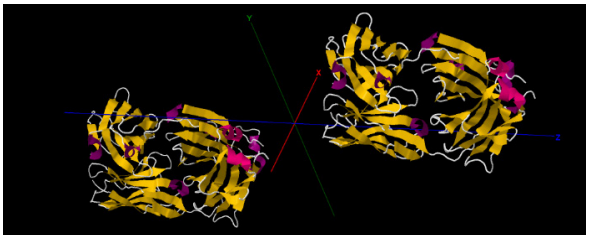

Figure 2: Molecular structure of cetuximab

Encorafenib has a molecular weight of 540.011 g/mole and its chemical formula is $C_{22}H_{27}ClFN_7O_4S$, as shown in Figure 1. Cetuximab has a protein chemical formula of $C_{6484}H_{10042}N_{1732}O_{2023}S_{36}$ with a protein average weight of 145781.6 Da, as shown in Figure 2.

Several analytical techniques for quantifying encorafenib and cetuximab have been published.[14-21] Currently, there is no known method for quantifying both medicinal drugs simultaneously using reverse-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals and Reagents

Samples of encorafenib and cetuximab were supplied as the reference material by Cipla Pharmaceuticals in Vijayawada. All compounds, including LCMS-grade acetonitrile, triethylamine and ortho phosphoric acid, were procured from the chemical division of Merck located in Mumbai.

HPLC Instrumentation

The investigation was carried out utilizing a HPLC apparatus comprised of a Waters e 2695 Shimadzu column, a photodiode array (PDA) detector, and Empower software version 2.0 as its driver. The chromatographic procedure involved the utilization of a Waters X-Terra RP-18 column measuring 250 x 4.6 mm and employing a particle size of 5 µL. The separation procedure was executed utilizing a mobile phase comprising a volumetric ratio of 40:60, which was composed of acetonitrile and 0.1% TEA pH-2.5/OPA. The eluents were identified by employing a UV detector set at 240 nm, while the flow rate was controlled at 1.0 mL/min and the injection volume was 10 µL.

Establishment of a Standard Solution

Carefully measure and transfer 75 mg of encorafenib and 5 mg of cetuximab into a 100 mL volumetric flask that has been thoroughly cleaned and dried in order to provide a working reference. Use sonication and a diluent to completely break it up. The volume can then be adjusted to the appropriate number using the same solvent. This is a stock management solution. Furthermore, add 5 mL of the previously indicated stock solutions to a 50 mL volumetric flask using a pipette. Gradually add diluent until the desired level is obtained. Encorafenib has a concentration of 75 ppm, whereas cetuximab has a concentration of 2 ppm.

Sample Solution Preparation

Accurately measure and transfer 202 mg of encorafenib and 1-mL of cetuximab into a dry, sterile volumetric flask. Dilute it with a solvent and sonicate it for up to 30 minutes to break it up. Then, centrifuge it for 30 minutes to break it up fully, and use the same liquid to make the volume up to the mark. After that, a 0.45-micron injection filter (Stock solution) is used to clean it. Next, fill a 50 mL volumetric jar with 5 mL of the aforementioned stock solutions using a pipette. Diluents can be added till the mark is attained. About 75 ppm of encorafenib and 2 ppm of cetuximab

Analytical Method Validation

Validation, according to the FDA, is "documented evidence that relies on a precise procedure to consistently generate a predefined quantity and quality of product." Validation's primary purpose is to establish a formal system for production and process control that will ensure that the products are what they claim or are represented to be. Validation attempts to show that a specified analytical process is competent for a wilful reason. Because routine quality control could not always assure a product's quality control owing to the small number of samples tested, validation must rather offer confirmation that such a system and perhaps product will fulfill pre-determined specifications or characteristics, giving a high degree of confidence that the very same quality will indeed be infused through each group from beginning to end.

System Fitness

Whenever a system is applied to an analysis, system fitness tests are performed. In order to determine whether a system is fit, we need to determine whether its RSD, theoretical plates, tailing factor, and resolution do not exceed a specified limit of its precession, given the results obtained at different time intervals.

Specificity

Specificity refers to the capability of analyte identification unequivocally in the existence of potentially interfering elements like matrix, impurities and, degradants, etc. Identification of analyte, purity tests, and testing assays are the three facets of this definition.

Linearity

For the purpose of assessing linearity, a total of six concentrations were collected from the baseline. This assessment was conducted by visually examining a plot that displayed the signals in relation to the analyte concentration. The test data were used to do statistical analysis by constructing a regression line using the least squares approach.

Accuracy

Whether a procedure is accurate is determined by the covenant among a traditionally recognized value as true or acknowledged as reference besides found value or it can be demarcated as adequacy amongst found as well as the most probable, true value.

Precision

Precision pertains to the extent of agreement among measurements performed utilizing several samples of a homogenous sample under prescribed conditions in an analytical technique.

Repeatability

To ensure accurate evaluation of repeatability and precision in similar operating conditions over a short period of time, it is advisable to perform at least six determinations at 100% concentration.

Intermediate precision

This is determined in the premise of the same laboratories but separate days, various observers, varied apparatus etc.

Forced degradation studies

An attempt was made to partially degrade the encorafenib and cetuximab sample by putting it through a series of forced degradation settings. In order to determine whether forced degradation is appropriate for products of degradation, researchers have conducted a number of studies.

- *Acid degradation*

A volumetric flask is used to combine 5 mL of sample stock solution with 1-mL of 1N HCl. The mixture is then allowed to settle for a duration of 15 minutes. To complete the reaction, add 1-mL of NaOH and then dilute the mixture to the desired concentration using diluents. Allow the reaction to proceed for 15 minutes. The solution was subsequently pumped into the HPLC machine.

- *Alkali degradation*

A 10 mL volumetric flask was filled with 1-mL of the sample stock solution and 1-mL of 1N NaOH. The mixture was then allowed to sit for 15 minutes. After the reaction is finished, add 1-mL of 1N HCl and use diluents to dilute it to the appropriate concentration. The solution was then injected into the HPLC device.

- *Peroxide degradation*

About 1-mL of the sample stock solution was diluted with 30% hydrogen peroxide in a 10 mL volumetric flask. After that, a solution was injected into the HPLC apparatus.

- *Reduction degradation*

The sample stock solution was diluted with 30% sodium bisulfate in a 10 mL volumetric flask, using 1-mL of the stock solution. Following that, the solution was introduced into the HPLC machine.

- *Thermal degradation*

A sample containing 500 mg of encorafenib and 10 mL of cetuximab was subjected to heat in an oven set at 105°C for a duration of 3 hours. After the exposure, the sample was analyzed. A sample containing 202 mg of encorafenib and 1-mL of cetuximab was transferred into a 100 mL volumetric flask. To dissolve, add 70 mL of diluents and sonicate. Then, dilute the solution to the desired volume using diluent. To prepare the solution, take 5 mL of the previously mentioned solution and add it to a 50 mL volumetric flask. Then, fill the flask with diluents until it reaches the mark. The solution mentioned above is injected into the HPLC system.

- *Photolytic degradation*

A sample containing 500 mg of encorafenib and 10 mL of cetuximab was placed in a photostability chamber for a duration of 3 hours. After the allotted time, the exposed sample was analyzed. A sample containing 202 mg of encorafenib and 1-mL of cetuximab was transferred into a 100 mL volumetric flask. To dissolve, add 70 mL of diluents and sonicate. Then, dilute the mixture to the desired volume using diluent. To prepare the solution, take 5 mL of the aforementioned solution and add it to a 50 mL volumetric flask. Then, fill the flask with diluents until it reaches the mark. The solution mentioned above is introduced into the HPLC system.

- *Hydrolysis degradation*

To prepare the solution, carefully transfer 5 mL of the provided solution using a pipette into a 50 mL volumetric flask. Then, add 3 mL of HPLC water to the flask. Next, heat the flask for a duration of 30 minutes. Finally, allow the flask to cool down to room temperature. After cooling, the solution is diluted to the desired volume with a diluent. The solution mentioned above is injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatographic Optimization

After carrying out a series of experimental protocols, it was noted that the mobile phase, which comprised 40:60 of acetonitrile and 0.1% TEA pH-2.5/OPA, displayed a peak characterized by desirable theoretical plate count, resolution, and tailing factor. Therefore, this procedure was optimised and validated. The optimized chromatogram is depicted in Figure 3.

Method's Validation

System suitability parameters

The analytical processes involve conducting tests to determine the compatibility of the system. Table 1 displays the results that have been compiled.

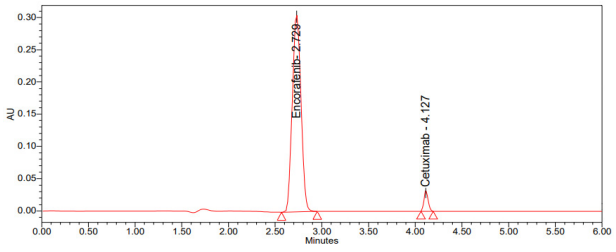

Figure 3: Optimized chromatogram encorafenib and cetuximab

Table 1: System suitability parameters for encorafenib & cetuximab

Parameter	*Encorafenib*	*Cetuximab*
Retention time	2.729	4.127
Plate count	13809	7465
Tailing factor	1.04	1.15
Resolution	----	7.39
%RSD	0.17	0.35

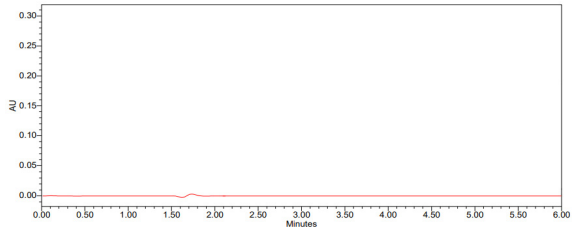

Figure 4: Blank chromatogram

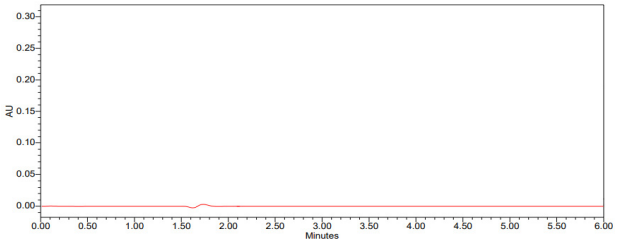

Figure 5: Placebo chromatogram

Specificity and selectivity

The procedure's specificity and selectivity were assessed by looking for interference peaks in the chromatograms of placebo and blank samples. In the retention time ranges, the HPLC chromatograms for the drug matrix (a mix of the Drug and placebos) revealed almost no interference peaks. As a result, the HPLC approach presented in this study was restricted. Figures 4 and 5 show the chromatograms of the placebo solution.

Linearity

Six concentration levels within the designated ranges were used to evaluate the linearity of the analytical method: 1.25 to 7.50 µg/mL for cetuximab and 18.75 to 112.50 µg/mL for encorafenib. The regression line seen in Figures 6 and 7 has had its slope, y-intercept, and regression coefficient calculated. Table 2 displays the results of cetuximab and encorafenib.

Accuracy

Samples were prepared using the conventional addition process, which ensured three distinct degrees of precision. Three injections were taken for each degree of accuracy, and the average% was calculated. The recovery rates for encorafenib and cetuximab were 99.7 and 99.8% respectively. The findings are displayed in Tables 3 and 4.

Precision

From a single volumetric flask of the working standard solution, six injections were made, and the areas that were obtained were

Table 2: Results of linearity for encorafenib and cetuximab

S. No.	*Encorafenib*		*Cetuximab*	
	Conc (µg/mL)	*Peak area*	*Conc (µg/mL)*	*Peak area*
1	18.75	495068	1.25	33215
2	37.50	988745	2.50	65984
3	56.25	1475496	3.75	99647
4	75.00	1976477	5.00	132458
5	93.75	2414459	6.25	168952
6	112.50	2965454	7.50	193078
Regression equation	y = 26138.81x +3363.14		y = 26205.20x + 778.21	
Slope	26138.81		26205.20	
Intercept	3363.14		778.21	
R^2	0.99983		0.99935	

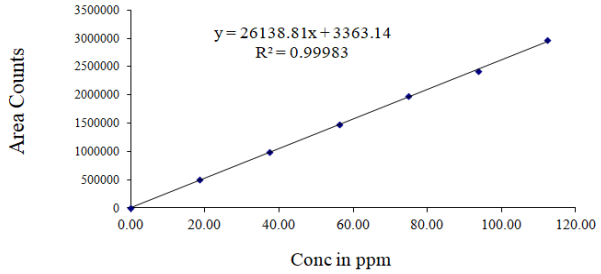

Figure 6: Calibration curve for encorafenib

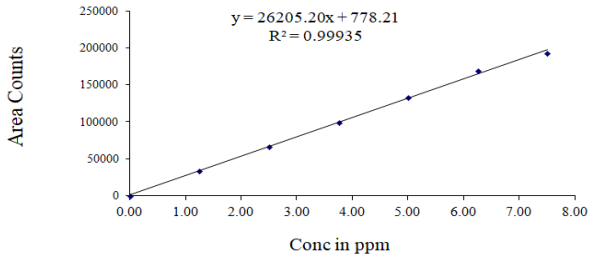

Figure 7: Calibration curve for cetuximab

Table 3: Encorafenib accuracy findings using the RP-HPLC

%Concentration	*Area*	*%Recovery*	*Mean recovery*
50	991744	100.03	
100	1969112	99.3	99.7
150	2963814	99.6	

Table 4: Cetuximab encorafenib accuracy findings using the RP-HPLC

%Concentration	*Area*	*%Recovery*	*Mean recovery*
50	65620	99.4	
100	132009	100.0	99.8
150	198072	100.0	

Table 5: Encorafenib with cetuximab precision

S. No	*Concentration encorafenib (µg/mL)*	*Area of encorafenib*	*Concentration of cetuximab (µg/mL)*	*Area of cetuximab*
1.	75	1985642	5	131739
2.	75	1980647	5	131899
3.	75	1982633	5	132774
4.	75	1987698	5	132096
5.	75	1978677	5	131435
6.	75	1984684	5	132187
Mean	1983330		132022	
S.D	3334.45		455.63	
%RSD	0.17		0.35	

shown in Table 5. For two drugs, the average area, standard deviation, and %relative standard deviation were found. Encorafenib had an RSD value of 0.17% and cetuximab had an RSD value of 0.35%. In this case, the system precision passed because the precision was less than 2.

Robustness

The %RSD of the test for encorafenib cetuximab was below 2.0 in each of the purposely adjusted chromatographic settings. The strategy was robust since the features of the system appropriateness did not change when the circumstances changed. The results are displayed in Tables 6 and 7.

LoD and LoQ (µg/mL)

To obtain encorafenib & cetuximab quantization limits, a signal/noise ratio strategy was exploited. As encorafenib & cetuximab quantization limits, the concentration of encorafenib & cetuximab signal/noise ratio of 10 was exploited. The quantitation limit was gauged as 0.75 µg/mL for encorafenib and 0.05 µg/mL for cetuximab.

To obtain encorafenib & cetuximab detection limits, a signal/noise ratio strategy was exploited. As encorafenib & cetuximab detection limits, the concentration of encorafenib & cetuximab at a signal/noise ratio of 3 was exploited. The detection limit was gauged as 0.225 µg/mL for encorafenib and 0.015 µg/mL for cetuximab.

Forced degradation studies

The stress research findings indicate that both medications and their combined drug product demonstrate stability under alkali, oxidative, and humidity-stressed circumstances. However, they exhibit instability under acid, alkali peroxide conditions (Table 8). Therefore, it is recommended that both individual pharmaceuticals and their combination drug product be stored in a properly sealed container to protect them from heat and light. The chromatograms of the stressed sample (exposed to acid, alkali, and peroxide) clearly demonstrate that the peaks of the active analytes can be differentiated from the degraded product, indicating that the approach is selective. This is shown in Figures 8, 9, and 10.

Table 6: Robustness results of encorafenib by HPLC

Parameter	*Condition*	*Retention time (minutes)*	*Peak area*	*Plate count*	*%RSD*
	Less flow (0.9 mL)	2.919	1782963	13963	0.56
Flow rate change (mL/min)	Actual (1-mL)	2.729	1985642	13809	0.17
	More flow (1.1 mL)	2.406	2155693	13789	0.93
Organic phase change	Less org (36:64)	2.858	1530456	13955	0.68
	Actual (40:60)	2.731	1980647	13825	0.17
	More org (44:56)	2.573	2224536	13726	0.43

Table 7: Robustness results of cetuximab by RP-HPLC

Parameter	*Condition*	*Retention time (minutes)*	*Peak area*	*Plate count*	*%RSD*
	Less flow (0.9 mL)	4.235	111128	7576	0.95
Flow rate change (mL/min)	Actual (1-mL)	4.127	131739	7465	0.35
	More flow (1.1 mL)	3.714	146875	7358	0.73
Organic phase change	Less org (36:64)	4.324	108351	7520	0.80
	Actual (40:60)	4.126	131899	7498	0.35
	More org (44:56)	3.820	157482	7319	0.71

Table 8: Results of forced degradation for cetuximab and encorafenib

Degradation	*Encorafenib*	*Cetuximab*
Control	0	0
Acid	13.3	10.7
Alkali	12.9	12.0
Peroxide	15.0	13.2
Reduction	2.6	1.8
Thermal	3.0	4.6
Photolytic	3.7	3.7
Hydrolysis	1.7	2.2

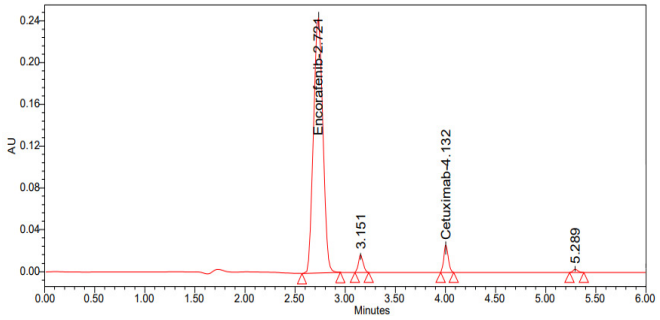

Figure 8: Acid degradation chromatogram

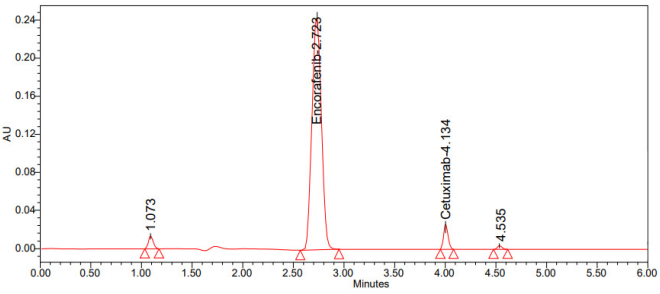

Figure 9: Alkali degradation chromatogram

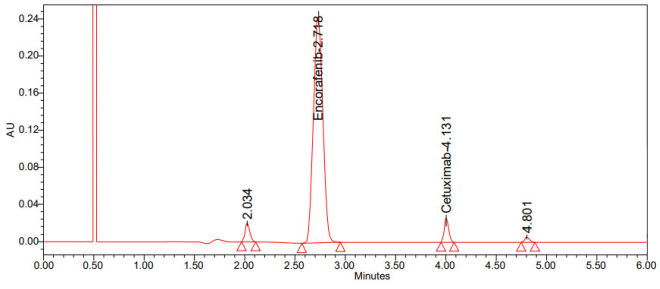

Figure 10: Peroxide degradation chromatogram

CONCLUSION

Encorafenib and cetuximab are two drugs that have been described as novel, and the approach is also innovative, with a lot of support for the created method in their validation. The suggested HPLC technique provided enough resolution for accurate chemical quantification. The accuracy and repeatability data are deemed acceptable based on the statistical analysis conducted on the experimental findings. For routine drug research analysis, the well-established chromatographic approach can be applied.

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