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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF COMBINED ANTINEOPLASTIC AGENTS BY RP-HPLC

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Abstract

A rapid and reliable RP-HPLC method was developed and validated for the simultaneous quantification of Encorafenib and Cetuximab in bulk and pharmaceutical dosage forms. Chromatographic separation was achieved using a Waters X-Terra RP-18 column with a mobile phase of Acetonitrile and 0.1% TEA buffer (pH 2.5) in a 40:60 ratio, at a flow rate of 1.0 mL/min, and detection at 240 nm. The method was validated as per ICH guidelines for system suitability, specificity, linearity, accuracy, precision, robustness, and stability-indicating capability. Linearity was observed over a range of 18.75–112.50 µg/mL for Encorafenib and 1.25–7.50 µg/mL for Cetuximab. Recovery values were within acceptable limits and % RSD was consistently below 2%, indicating good accuracy and precision. Forced degradation studies under acidic, basic, oxidative, photolytic, and thermal conditions confirmed the method's specificity and stability-indicating nature. The method proved to be robust under minor variations in flow rate and mobile phase composition. Overall, the developed method is simple, accurate, and suitable for routine quality control and stability studies of Encorafenib and Cetuximab in combined formulations

Keywords: Encorafenib, Cetuximab, Method development, Validation.

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INTRODUCTION

Encorafenib is a selective small-molecule inhibitor of BRAF kinase, specifically targeting the mutated BRAF V600E and V600K variants found in several cancers [1]. These mutations cause constitutive activation of the MAPK signaling pathway,driving uncontrolled cell proliferation and tumor growth. By inhibiting BRAF kinase, Encorafenib disrupts downstream signaling in the MAPK pathway, including the MEK and ERK kinases, thereby reducing tumor cell proliferation and inducing apoptosis in cancer cells harboring the BRAF mutation. Encorafenib is often used with other targeted agents, such as MEK inhibitors, to enhance its therapeutic efficacy and overcome resistance mechanism [2]. Cetuximab is a drug that blocks EGFR and is employed in the treatment of metastatic colorectal cancer and cancers of the head and neck [3]. The molecular weight of Cetuximab is approximately 152 kDa [4]. Cetuximab is a monoclonal antibody that ischimeric (mouse/human) and binds to and inhibits EGFR [5].

MATERIALS AND METHODS

Chemicals and Reagents

The study employed API samples of Cetuximab and Encorafenib obtained from the Pharma Life Research facility in Hyderabad, Telangana. Rankem Chemicals,Haryana, India, provided the orthophosphoric acid (OPA) and acetonitrile (ACN) (HPLC-grade and AR-grade chemicals).

Chromatographic Method and System Details

The RP-HPLC analysis was executed using a Waters Alliance 2695 system with a UV detector, controlled via Empower 2 software. The stationary phase was a Waters X-Terra RP-18 column (250 × 4.6 mm, 5 µm). A mobile phase mixture of Acetonitrile and 0.1% Triethylamine buffer (adjusted to pH 2.5) in 40:60 ratio was used. A 10 µL sample was injected, the flow rate was set to 1.0 mL/min, and UV detection was performed at 240 nm over a total run time of 6 minutes.

Solution Preparation

A standard solution was prepared by weighing 75 mg of

Encorafenib and 5 mg of Cetuximab into a 100 mL volumetric flask, dissolving with diluent, sonicating, and bringing to volume. For the sample, 202 mg of Encorafenib and 1 mL of Cetuximab were treated similarly, followed by a 30-minute sonication and centrifugation. After filtration (0.45 µm), 5 mL from each was diluted to 50 mL, resulting in working concentrations of 75 ppm and 5 ppm, respectively.

System Suitability

System suitability was evaluated by injecting standard solutions into the HPLC system and measuring parameters such as retention time, theoretical plate count, tailing factor, resolution, and % relative standard deviation (%RSD). These metrics ensured that the system performance was acceptable for accurate and precise analysis [6].

Specificity

Specificity was assessed by analyzing blank, placebo, and standard solutions to verify the absence of interfering peaks at the retention times of Encorafenib and Cetuximab. This test confirmed that the method could selectively quantify both analytes without interference from excipients or other components [7].

Linearity

Linearity was established by preparing a series of standard solutions across a range of concentrations for each drug. The linear concentration range was selected to cover expected sample concentrations. Calibration curves were plotted using peak area versus concentration, and regression analysis was performed to calculate the correlation coefficient and other parameters [8].

Accuracy and Precision

The developed method was validated for accuracy, precision, and sensitivity. Accuracy was confirmed through recovery studies conducted by spiking pre-analyzed samples of Encorafenib and Cetuximab at 50%, 100%, and 150% concentration levels, with each level analyzed in triplicate. The mean percent recovery values supported the method's reliability. Precision was assessed at three levels: system, method, and intermediate precision, with results expressed as %RSD of peak areas. Intermediate precision accounted for variations between analysts and days. [9-11].

Robustness and Assay

Method robustness was evaluated by applying slight but deliberate variations in chromatographic conditions, particularly flow rate (±0.1 mL/min) and mobile phase ratios. The method's reliability was confirmed by consistent retention times, resolution, peak areas, and system suitability parameters under these changes. For the assay, individual injections of standard and sample solutions were analyzed using HPLC. The drug content in the sample was quantified by referencing the calibration curve derived from the standard peak areas, confirming the method's applicability for routine quantitative analysis [12, 13].

Forced Degradation Studies

Forced degradation studies were carried out under various ICH-recommended conditions-acidic and basic hydrolysis, oxidative stress, heat, light, and aqueous environments-to evaluate whether the developed RP-HPLC method could effectively separate the active drug from its degradation products. Peak purity was assessed to ensure the specificity of the method.[14-17].

RESULTS AND DISCUSSION

System Suitability

Encorafenib has a retention time of 2.729 minutes, with a plate count of 13,809 and a tailing factor of 1.04, indicating excellent column efficiency and symmetrical peak shape. Cetuximab showed a retention time of 4.127 minutes, with a plate count of 7,465 and a tailing factor of 1.15, demonstrating good peak integrity. The resolution between the two drugs is 7.39, ensuring clear separation. The %RSD values are low (0.17% for Encorafenib and 0.35% for Cetuximab), indicating precise and reproducibleresults.

Specificity

Figures 01 demonstrate the specificity of the chromatographic method for Encorafenib and Cetuximab, no interfering peaks are observed at the retention times corresponding to Encorafenib (2.729 min) and Cetuximab (4.127 min). In the optimized chromatogram distinct peaks for both drugs are well- separated and free from interference. This confirms that the method is specific for accurately detecting and quantifying Encorafenib and Cetuximab without interference from other

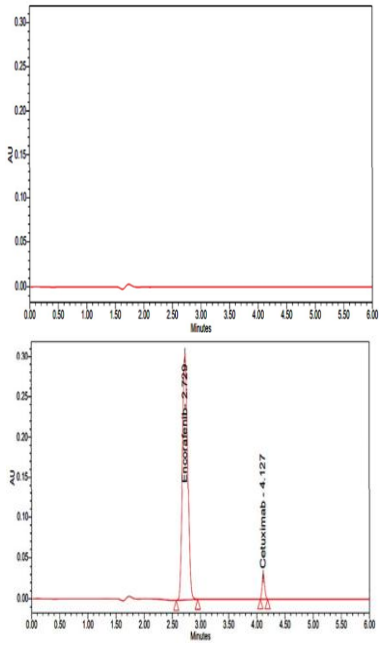

Fig 01: Chromatogram of blank and Optimized chromatogram

Linearity

The proposed method is found to be linear in the concentration range of 18.75 to 112.50µg/ml and 1.25 to 7.50µg/ml for Encorafenib and Cetuximab respectively. The regression equations for Encorafenib and Cetuximab are found to be y=26138.81x+3363.14 and y=26205.20x+778.21, respectively. The linearity data of Encorafenib and Cetuximab. From the results it is observed that Encorafenib and Cetuximab has correlation coefficients (R^2) of 0.99983 and 0.99935, indicatingexcellentlinearity and also confirming strong linearityThe calibration curves of Encorafenib and Cetuximab are shown in Figure 02.

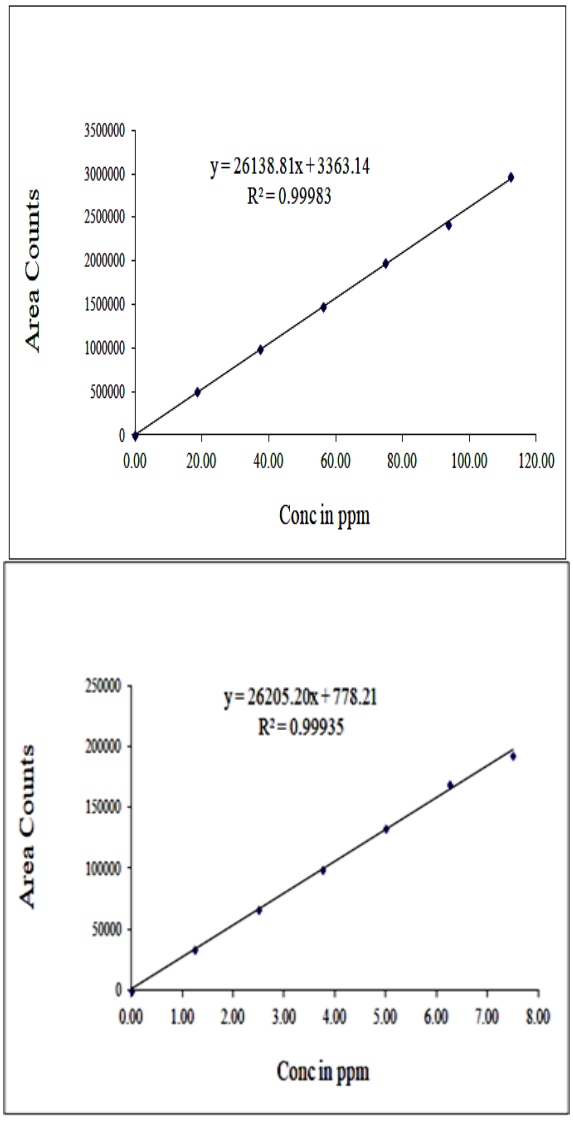

Fig 02: Calibration curve of Encorafenib and Cetuximab

Accuracy

The recovery studies for Encorafenib and Cetuximab at 50%, 100%, and 150% concentration levels showed mean recoveries ranging from 98.8% to 100.4% for Encorafenib and 99.1% to 101.3% for Cetuximab. The %RSD values for both drugs were below 1.5%, indicating excellent precision and accuracy of the method across all levels (Table 01).

Table 01: Accuracy data of Encorafenib and Cetuximab

	Encorafenib			Cetuximab		
%Conc	%Recovery	%Mean Recovery	%RSD	% Recovery	%Mean Recovery	%RSD
50 %	99.9	99.9	0.31	99.4	99.5	0.24
	100.4			99.7		
	99.8			99.1		
100 %	99.1		0.52	99.6		1.21
	100.0			101.3		
	98.8			99.1		
150 %	99.5		0.58	99.3		0.85
	100.3			99.8		
	99.1			100.9		

Precision

The method precision and intermediate precision for Encorafenib and Cetuximab demonstrated consistent results, with %RSD values below 1% for both drugs, indicating excellent reproducibility. The average peak areas for Encorafenib were 1,979,788 (method) and 1,977,336 (intermediate), while for Cetuximab they were 132,290 and 132,356, respectively. The low standard deviation and %RSD values confirm the reliability of the analytical method under normal and varied conditions (Table 02).

Table 02: Method precision and Intermediate Precision

	Method Precision		Intermediate Precision	
S. No.	Area for Encorafenib	Area for Cetuximab	Area for Encorafenib	Area for Cetuximab
1	1982574	130824	1982214	132524
2	1977785	131563	1960365	133563
3	1993652	132478	1981475	131478
4	1958582	132875	1984350	130975
5	1981165	133582	1992158	132582
6	1984968	132415	1963451	133015
Average	1979788	132290	1977336	132356
Standard	11682.528	973.667	12574.754	964.207

Deviation				
%RSD	0.59	0.74	0.64	0.73

Robustness

The robustness studies for Encorafenib and Cetuximab, demonstrate that the developed RP-HPLC method remains reliable under deliberate variations in chromatographic conditions. For Encorafenib, changes in flow rate (±0.1 mL/min) and organic phase composition (±4%) resulted in slight shifts in retention time and peak area, but parameters such as tailing factor (1.04–1.08), resolution, plate count, and %RSD (≤0.93%) remained within acceptable limits, indicating method stability. Similarly, Cetuximab showed consistent performance across varied conditions, with retention time ranging from 3.71 to 4.32 minutes, resolution maintained between 6.25 and 7.75, tailing factor within 1.15–1.18, and %RSD values all below 1%, confirming the robustness and reliability of the analytical method for both analytes.

Assay

The results are shown in table 11. From the results, it is observed that the test yielded 99.5% and 100.2% result, indicating excellent accuracy and conformity with the label claim (Table 03)

Table 03: Assay of Encorafenib and Cetuximab

Drug	Avg sample area (n=5)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label amount (mg)	Amount found (µg/ml)	% assay
Encorafenib	1980893	75	75	75	74.908	99.5
Cetuximab	133089	5	5	5	5.04	100.2

Forced Degradation Studies

The forced degradation studies (Table 04) revealed that both Encorafenib and Cetuximab were susceptible to acidic, alkaline, and oxidative conditions, with maximum degradation observed under peroxide stress (15.0% for Encorafenib and 13.2% for Cetuximab). In contrast, minimal degradation was noted under reduction, thermal, photolytic, and hydrolytic conditions, with degradation percentages below 5% for both drugs. The purity angle in all stress conditions remained well below the purity threshold, confirming peak purity and the stability-indicating nature of the method. The % assay values ranged from 85.0% to 100%, indicating that the method can accurately quantify the drug even in the presence of degradation products. Overall, the method was proven to be selective and robust under various stress conditions.

Table 04: Forced Degradation results for Encorafenib and Cetuximab

	Area	% Assay	% Deg	Area	% Assay	% Deg
Control	1984017	100	0	131995	100	0
Acid	1719478	86.7	13.3	117923	89.3	10.7
Alkali	1727364	87.1	12.9	116184	88.0	12.0
Peroxide	1685877	85.0	15.0	114572	86.8	13.2
Reduction	1930936	97.4	2.6	129564	98.2	1.8
Thermal	1922906	97.0	3.0	125937	95.4	4.6
Photolytic	1908747	96.3	3.7	127103	96.3	3.7
Hydrolysis	1948794	98.3	1.7	129124	97.8	2.2

CONCLUSION

A novel and robust RP-HPLC method is meticulously optimized, ensuring high accuracy, precision, and reproducibility. The method demonstrates excellent linearity with correlation coefficients close to 1.0 for both drugs across their respective concentration ranges. Stability studies under various stress conditions—acidic, alkaline, oxidative, thermal, photolytic, and hydrolytic—established the method's reliability as a stability-indicating approach. Sensitivity parameters such as LOD and LOQ highlight the method's capability to detect and quantify low concentrations effectively. Validation data, including system precision, repeatability, and intermediate precision, confirm its suitability for routine pharmaceutical analysis. Overall, this method provides a significant advancement in the simultaneous analysis of Encorafenib and Cetuximab, making it a valuable tool for quality control and research applications.

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Nil

CONFLICT OF INTEREST

Authors are declared that no conflict of interest.

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INFORMED CONSENT AND ETHICAL STATEMENT

Not Applicable

AUTHOR CONTRIBUTION

Jhansi Rani Sriramula conceptualized and designed the study, performed the RP-HPLC method development and validation, and interpreted the analytical data. Shivaraj assisted in method optimization, statistical

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