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Analytical Method Development and Validation for the Simultaneous Estimation of Evinacumab Injection Formulation by RP-HPLC

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Abstract: *This paper describes in detail the RP-HPLC method used to evaluate the content of evinacumab (EVC) in combination with evkeeza injections. The mobile phase used in the assay is a mixture of potassium dihydrogen phosphate and methanol in an isocratic ratio of 70:30 (v/v) on a column Shimpac solar C18, 150*4.6, 5µm column. The flow rate was 1.0 ml/min, isocratic. At 226 nm, the quantification and detection of EVC were done concurrently. It was discovered that the response obtained was linear over the concentration range of 75-225 µg/ml, and the estimated RT in minutes for EVC was 4.063. 1.805 µg/ml was the LOD and 6.017 µg/ml was the LOQ. For the EVC assay, precision was 0.3% RSD and accuracy was 100%.*

Keywords: Evinacumab, Simultaneous estimation, ICH guidelines.

I. INTRODUCTION

Evinacumab is a monoclonal antibodythat targets angiopoietin-like protein 3 (ANGPTL3) and is used with other lipid-lowering treatments to treat homozygous familial hypercholesterolemia. [1] Monoclonal antibodies are frequently broken down into smaller peptides and amino acidsthrough catabolic processes. [2]

Evinacumab 38 is a new monoclonal antibody that targets angiopoietin-like protein 3 (ANGPTL3) and is the first drug of its kind. ANGPTL proteins have diverse physiological activities, such as controlling lipid metabolism, which makes them attractive candidates for therapeutic interventions in contemporary medicine.[3] Loss-of-function mutations in ANGPTL3 lead to decreased lipid levels and a decreased susceptibility to cardiovascular illnesses. Heightened ANGPTL3 activity is linked to an elevated susceptibility to cardiovascular illnesses, leading to the development of a medication that selectively targets ANGPTL3. [4]

Evinacumab, an ANGPTL3 inhibitor, received FDA approval in February 2021 for the treatment of homozygous familial hypercholesterolemia (HoFH) and is sold under the brand name Evkeeza for injection. Evinacumab offers a unique and additional treatment option for Homozygous Familial Hypercholesterolemia due to its innovative method of action, distinguishing it from other lipid-lowering medications. [5]Evinacumab was authorized by Health Canada in September 2023 and by the European Medicines Agency (EMA) in December 2023 for the identical therapeutic use. [6] Peak efficiency HPLC is a form of column chromatography in which the mobile phase moves quickly across the column. The analytical time is decreased by 1-2 orders of magnitude in comparison to conventional column chromatography. Using smaller absorbent or support particles can significantly improve column efficiency. [7-15] The graphic displays the primary elements of the HPLC. Upon extensive literature, till now no method has been reported for estimation of EVINACUMAB. In the present research work an attempt has been made to develop and validate an RP-HPLC method for estimation of Evinacumab in bulk and in pharmaceutical formulations.

II. MATERIALS AND METHODS

2.1 Instrumentation:

Chromatography was performed with Alliance waters 2696 HPLC with high speed auto sampler, column oven, degasser and 2998 PDA detector to provide a compact and convenient for LC with class Empower 2 software.

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2.2 Chemicals and Reagents:

The reference samples of Evinacumab were provided as gift samples. HPLC grade Acetonitrile, HPLC grade Methanol, HPLC grade water and all other chemicals were obtained from Rainbow Pharma Training Lab, Hyderabad. Commercial injection Evkeeza™ (evinacumab-dgnb) Injection [Dosage: 345 mg/2.3 mL (150 mg/mL)] were provided by Rainbow Pharma Training Lab, Hyderabad.

2.3 Chromatographic Conditions:

The mobile phase consisted of Buffer and methanol taken in the ratio of 70:30 at a flow rate of 1 mL/min. SHIMPAC SOLAR, C18, 150mm x 4.6 mm, 5µ particle size was used as the stationary phase. EVINACUMAB have different ɟ max, but considering the chromatographic parameter, sensitivity and selectivity of method for both drugs, 226 nm was selected as the detection wavelength for PDA detector.

2.4 Preparation of standard stock solution and standard solution:

A stock solution of EVC was made by dissolving 150 milligrams of EVC in 100 ml of the chosen diluent, yielding a concentration of 1500 µg/ml.

An EVC solution was created by diluting 1 ml of a concentrated solution with a concentration of 1500 µg/ml in 10 ml of a diluent to get a final concentration of 150 µg/ml.

LINEARITY EVC SOLUTIONS:

- Solution one for linearity with a concentration of 50 µg/ml, 0.5 ml of a stock EVC solution (concentration of 150 µg/ml) was mixed with 9.5 ml of the selected diluent.
- To prepare a solution with a concentration of 75 µg/ml, 0.75 ml of a stock EVC solution (concentration of 150 µg/ml) was mixed with 9.25 ml of a selected diluent.
- To prepare a solution with a concentration of 100 µg/ml, 1.0 ml of a stock EVC solution (concentration of 150 µg/ml) was mixed with 9.0 ml of a specified diluent.
- To prepare a solution with a concentration of 125 µg/ml, 1.25 ml of a stock EVC solution (concentration of 150 µg/ml) was mixed with 8.75 ml of a selected diluent.
- To prepare a solution with a concentration of 150 µg/ml, 1.50 ml of a stock EVC solution (concentration of 150 µg/ml) was mixed with 8.50 ml of a selected diluent.

2.5 Sample Preparation:

Calculate the EVC in Evkeeza injection by mixing 150 mg of Evinacumab injectable concentrate in a 100 ml clean, dry volumetric flask using sonication, and adjusting the volume. After filtration, 1 mL of the solution was transferred to a 10 mL volumetric flask. A diluent was added to get a final volume of 10 ml.

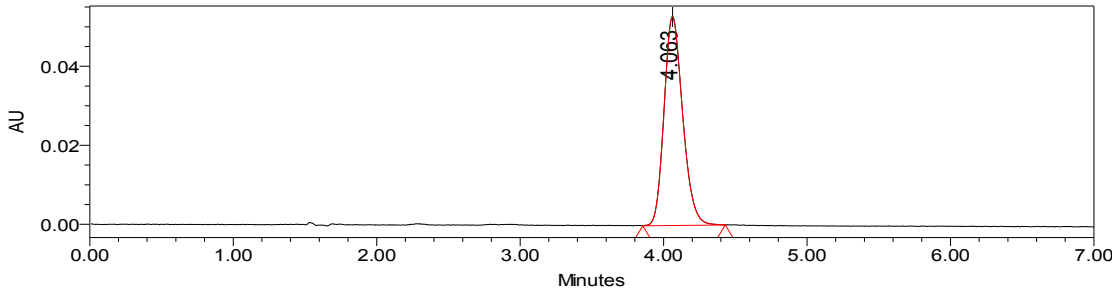

Figure 1: A typical Chromatogram of Evinacumab

III. RESULTS AND DISCUSSION:

3.1 Method development

Initially, an RP-HPLC method was employed to assess the content of evinacumab (EVC) in combination with evkeeza injections. The assay utilized a mobile phase comprising a mixture of potassium dihydrogen phosphate and methanol at

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pH 4.6, in an isocratic ratio of 70:30 (v/v), running on a Shimpac solar C18 column (150 × 4.6 mm, 5 µm). Both the column and sample temperatures were maintained at 25°C, with an injection volume of 10 µL. The flow rate was held constant at 1.0 mL/min in an isocratic mode. EVC quantification and detection were performed simultaneously at 226 nm. It was observed that the response exhibited linearity within the concentration range of 75-225 µg/mL, with an estimated retention time (RT) for EVC at 4.063 minutes. The limit of detection (LOD) was determined to be 1.805 µg/mL, while the limit of quantification (LOQ) stood at 6.017 µg/mL. Precision, expressed as the relative standard deviation (RSD), was found to be 0.3%, while accuracy was validated at 100% for the EVC assay.

3.2 Method Validation:

3.2.1 System Suitability and Specificity:

System suitability parameters, including the number of theoretical plates, peak tailing, retention time, and resolution factor, were assessed. The method's total runtime for eluting EVC is merely 7 minutes. The findings are presented in Table 1.

SYSTEM SUITABILITY PARAMETERS	*EVINACUMAB*
No of theoretical plates	*4734*
Tailing Factor	*1.19*
Resolution	*-*
RT	*4.063*
Mean Area	*478033*
%RSD	*0.4*

Table 1: System suitability investigation readings for Evinacumab

3.2.2 Linearity:

To maintain linearity, a concentration of 75 µg/ml, 112.5 µg/ml, 150 µg/ml, 187.5 µg/ml, 225 µg/ml of EVC was achieved by combining 0.5 ml, 0.75 ml, 1.0 ml, 1.25 ml, 1.50 ml of a EVC stock solution (with a concentration of 150 µg/ml) with 9.5 ml, 9.25 ml, 9.0 ml, 8.75 ml,8.50 ml of the selected diluent.

The collected data exhibits a reasonable degree of linearity for EVC analysis. The results obtained were shown in Table 2.

EVINACUMAB	
µg/ml Amount	*Response Peak Area*
75.00	*231712*
112.50	*355533*
150.00	*470509*
187.50	*589226*
225.00	*701718*

Table 2: Evinacumab response peak area and concentration.

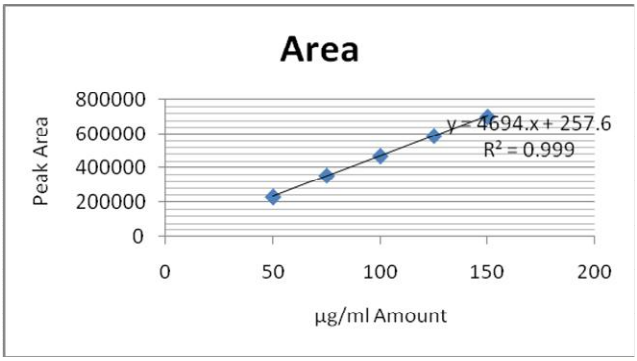

Figure 2: Evinacumab calibration curves

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3.2.3 Accuracy / Recovery:

Six injections were carried out using a 10 µL solution containing EVC (EVC– 150 µg/mL) on a C18 column measuring 150 x 4.6 mm with a particle size of 5 µm. The data was evaluated based on the parameters outlined in the "EVC ANALYSIS CONDITIONS" section. The EVC assay results were measured as percentages. Statistical measures such as the mean, standard deviation, and relative percent standard deviation were calculated for six EVC injections. The results confirmed the accuracy of the current EVC analytical methodology. The results obtained were shown in Table 3.

µg/ml Amount Considered	*µg/ml Amount Quantified*	*Assay Percent*	
EVINACUMAB			
74.250	*73.08*	*98*	*Mean percent assay*
74.250	*73.34*	*99*	*100*
74.250	*73.46*	*99*	*Standard assay*
148.500	*151.16*	*102*	*Deviation*
148.500	*148.58*	*100*	*0.2*
148.500	*148.20*	*100*	*RSD*
222.750	*223.40*	*100*	*0.2*
222.750	*222.27*	*100*	
222.750	*222.21*	*100*	

Table 3: Accuracy investigation readings for Evinacumb

3.2.4 Precision / Repeatability:

Six injections of an EVC solution, each with a volume of 10 microliters and a concentration of 150 micrograms per milliliter, were loaded onto a C18 column of 150 x 4.6 mm with a particle size of 5 micrometers. The data was assessed according to the settings specified in the "EVC ANALYSIS CONDITIONS" section. The climax of the EVC responses was identified. Standard deviation and relative percent standard deviation were calculated for the EVC response peak in six areas. The collected data validated the precision of the methodology for EVC analysis. The results obtained were shown in Table 4.

Response Peak Area	
EVINACUMAB	
471546	*Mean response peak area*
469631	*470957*
472161	*Standard response Deviation*
470511	*1180.4*
469680	*RSD*
472215	*0.3*

Table 4: Precision investigation readings for Evinacumab

3.2.5 Robustness:

The evaluation criteria for the EVC solution at a concentration of 150 µg/ml were slightly modified under the "EVC ASSAYING CONDITIONS" section. The device values were deemed suitable for EVC. The effectiveness of EVC analytical procedures was shown through the collecting of data. The results obtained were shown in Table 5.

Analytical conditions	*Modified Analytical conditions*	*Mean RT*
Flow rate	*0.9*	*3.251*
(1.0 ml/min)	*1.1*	*4.786*
Mobile phase composition	*60:40*	*4.636*
KH^2PO^4: Methanol (70:30)	*80:20*	*4.786*
PH optimization	*4.4*	*3.985*
(4.6 pH)	*4.8*	*3.974*

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nm optimization (226 nm)	*221*	*4.063*
	231	*4.060*

Table 5: Robustness investigation readings for Evinacumab

3.2.6 Selectivity:

The selectivity was assessed using the usual addition approach, which included calculating the percentage of EVC recovery. EVC concentrations of 50%, 100%, and 150% were added to the EVC stock solution at concentrations of 75 µg/ml, 150 µg/ml, and 225 µg/ml, respectively. The samples underwent three rounds of testing according to the parameters outlined in "EVC ASSAYING CONDITIONS." The results obtained were shown in Table 6.

µg/ml Amount Considered	*µg/ml Amount Quantified*	*Recovered Percent*	
50% additional			
74.250	*73.08*	*98*	*Mean percent recovered 99*
74.250	*73.34*	*99*	
74.250	*73.46*	*99*	
100% additional			
148.500	*151.16*	*102*	*Mean percent recovered 101*
148.500	*148.58*	*100*	
148.500	*148.20*	*100*	
150% additional			
222.750	*223.40*	*100*	*Mean percent recovered 100*
222.750	*222.27*	*100*	
222.750	*222.21*	*100*	

TABLE 6: Selectivity investigation readings for Evinacumab

3.2.7 Stability:

The EVC stability reports feature chromatograms produced by subjecting the samples to various conditions, such as exposure to 0.1 N HCl, 0.1 N NaOH, peroxide, sunlight, and a temperature of 105°C. The arrangement with EVC is exceptional and well-suited for substances that have been harmed. The results showed that the procedure is reliable and precise when doing EVC assessments.

EVC stability reports after investigated through exposing to 0.1 N HCl, 0.1N NaOH, peroxide, sun light and 105^{o}C given as below table 7.

Drug exposed	*EVC*	
	Response Area	*% Assay*
Acid	*422925*	*88.93*
Base	*440297*	*92.58*
Peroxide	*442820*	*93.11*
Heat	*423250*	*89.00*
Sunlight	*447370*	*94.07*
Untreated	*466567*	*98.11*

Table 7: Stability of EVC

3.2.8 LOD and LOQ:

LOD and LOQ for Evinacumab were 1.805 µg/ml and 6.017 µg/ml respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive.

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IV. CONCLUSION

A novel, precise, accurate, and straightforward HPLC method has been devised and validated for the concurrent determination of Evinacumab in injection formulations through RP-HPLC. This method boasts rapidity, accuracy, precision, and sensitivity, rendering it suitable for routine quality control assessments of injection-containing medications within QC laboratories and industrial settings.

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