VALIDATED RP - HPLC METHOD FOR THE ESTIMATION OF DENOSUMAB IN FORMULATION

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ABSTRACT

A simple, selective, linear, precise and accurate RP-HPLC method was developed and validated for rapid assay of Denosumab in tablet dosage form. Isocratic elution at a flow rate of 1.0ml/min was employed on a symmetry C18 (250x4.6mm, 5µm in particle size) at ambient temperature. The mobile phase consisted of methanol: water: O.P.A 90:10:01 (V/V/V). The UV detection wavelength was 233 nm and 20µl sample was injected. The retention time for Denosumab was 3.64 min. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method was successfully applied for routine analysis of Denosumab in tablet dosage form.

Key Words: Denosumab, RP-HPLC, UV detection, recovery, precise, 233 nm

INTRODUCTION

Denosumab[1] is a fully human monoclonal antibody for the treatment of osteoporosis, treatment induced bone loss, bone metastases,rheumatoid arthritis, multiple myeloma and giant cell tumor of bone.[2][3] It was developed by the company Amgen.[4] Denosumab is designed to target RANKL (RANK ligand), a protein that acts as the primary signal to promote bone removal. In many bone loss conditions, RANKL overwhelms the body's natural defense against bone destruction. It was approved by U.S. Food and Drug Administration (FDA) for use in postmenopausal women with risk of osteoporosis in June 2010, under the trade name Prolia,[5] and for the prevention of skeletal-related events in patients with bone metastases from solid tumors in November 2010, as Xgeva,[6] making it the first RANKL inhibitor to be approved by the FDA.[7] In the summer of 2011 clinical trials were investigating giant cell tumors, multiple myeloma with bone metastases, dosing, safety, and hypercalcemia of malignancy.[8] Bone remodeling is the process by which the body continuously removes old bone tissue and replaces it with new bone. It is driven by various types of cells, most notably osteoblasts, which secrete new bone, and osteoclasts,

which break it down. The role of osteocytes is not well understood. Precursors to osteoclasts, called pre-osteoclasts, express surface receptors called RANK, short for receptor activator of nuclear factor-kappa B. RANK is a member of the tumor necrosis factor receptor superfamily. RANK is activated by RANKL (the RANK-Ligand), which exists as cell surface molecules on osteoblasts. Activation of RANK promotes the maturation of pre-osteoclasts into osteoclasts. Denosumab inhibits this maturation of osteoclasts by binding to and inhibiting RANKL – this mimics the natural action of osteoprotegerin, an endogenous RANKL inhibitor, that presents with decreasing concentrations and perhaps avidity in patients suffering from osteoporosis, thus protecting bone from degradation and helping to counter the progression of the disease.[2]

EXPERIMENTAL

Chemicals and reagents

HPLC grade water and methanol were purchased from Merck Specalities Pvt. Ltd.

Instrumentation and analytical conditions

The analysis of drug was carried out on a PEAK HPLC system equipped with a reverse phase C18 column (250x4.6mm, 5µm in particle size), a LC-P7000 isocratic pump, a 20µl injection loop and a LC-UV7000 absorbance detector and running on PEAK Chromatographic Software version 1.06. Isocratic elution with methanol: water: O.P.A 90:09:01 (V/V) (P^H-5.2) was used at a flow rate of 1.0ml/min. The mobile phase was prepared freshly and degassed by sonicating for 5 min before use.

Stock and Working standard solutions

Accurately weigh and transfer 10mg of Denosumab working standard into a 10ml volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm nylon filter paper and finally 10µg/ml were prepared. The calibration curve was plotted with the five concentrations of the 2µg/ml - 12µg/ml working standard solutions. Calibration solutions were prepared daily and analyzed immediately after preparation.

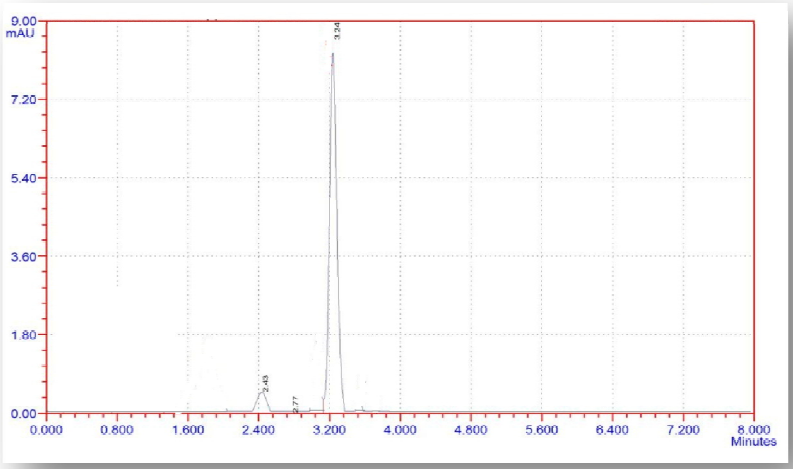

Fig 2: Typical chromatogram of Denosumab Formulation

VALIDATION PROCEDURE

The objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy, specificity, stability and system suitability. Standard plots were constructed with five concentrations in the range of 2µg/ml to 10µg/ml prepared in triplicates to test linearity. The peak area of Denosumab was plotted against the concentration to obtain the calibration graph. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method. The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from six replicate injections of freshly prepared Denosumab test solution in the same equipment at a concentration value of 100% (10µg/ml) of the intended test concentration value on the same day. The experiment was repeated by assaying freshly prepared solution at the same concentration additionally on two consecutive days to determine intermediate precision. Peak area of the Denosumab was determined and precision was reported as %RSD.

Method accuracy was tested (% recovery and %RSD of individual measurements) by analyzing sample of Denosumab at three different levels in pure solutions using three preparations for each level. The results were expressed as the percentage of Denosumab recovered in the samples. Sample solution short term stability was tested at ambient temperature (20 ± 10^0C) for three days. In order to confirm the stability of both standard solutions at 100% level and tablet sample solutions, both solutions protected from light were re-injected after 24 and 48 hours at ambient temperature and compared with freshly prepared solutions.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

Proper selection of the stationary phase depends up on the nature of the sample, molecular weight and solubility. The drug Denosumab in non-polar. Non-polar compounds preferably analyzed by reverse phase columns. Among C8 and C18, C18 column was selected. Non-polar compound is very attractive with reverse phase columns. So the elution of the compound from the column was influenced by polar mobile phase. Mixture of methanol, water and O.P.A was selected as mobile phase and the effect of composition of mobile phase on the retention time of Denosumab was thoroughly investigated. The concentration of the water, methanol, water and O.P.A were optimized to give symmetric peak with short run time (Fig.3).

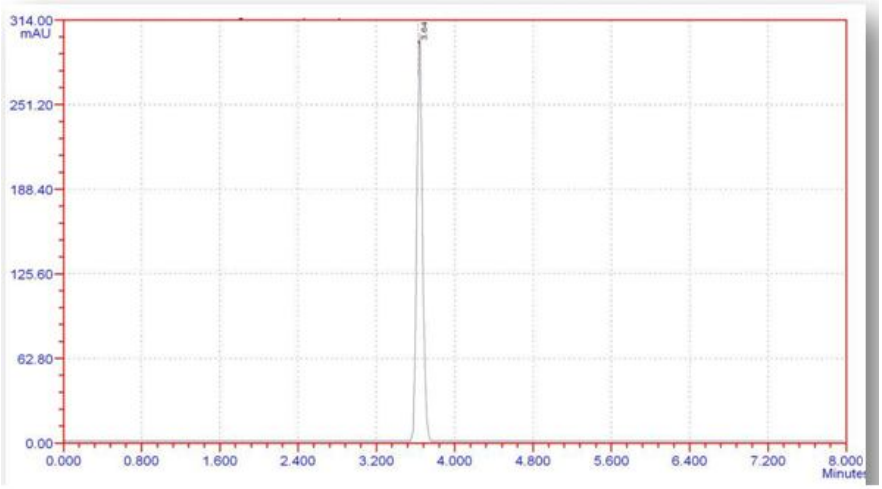

Fig 3: Typical chromatogram of Denosumab

Validation of method

Linearity

Five points graphs was constructed covering a concentration range 2-12 ppm (Three independent determinations were performed at each concentration). Linear relationships between the peak area signal of Denosumab the corresponding drug concentration was observed. The standard deviation of the slope and intercept were low. The statistical analysis of calibration is shown in Table 1.

S.No.	concentration	Area
1	2 ppm	4520
2	4 ppm	8279
3	6 ppm	12047
4	8 ppm	16351
5	10 ppm	20384
6.	12 ppm	24358

Table 1: Linearity of Denosumab

Precision

The validated method was applied for the assay of commercial tablets containing Denosumab. Sample was analyzed for five times after extracting the drug as mentioned in assay sample preparation of the experimental section. The results presented good agreement with the labeled content. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was showing that the equipment used for the study was correctly and hence the developed analytical method is highly repetitive. For the intermediate precision a study carried out by the same analyst working on the same day on two consecutive days indicated a RSD of 1.64. This indicates good method precision.

Stability

The stability of Denosumab in standard and sample solutions containing determined by storing the solutions at ambient temperature (20 ± 10^0C). The solutions were checked in triplicate after three successive days of storage and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 48 hrs, as during this time the results did not decrease below 98%. This denotes that Denosumab is stable and standard and sample solutions for at least 2 days at ambient temperature.

System suitability

The system suitability parameter like capacity factor, asymmetry factor, tailing factor and number of theoretical plates were also calculated. It was observed that all the values are within the limits (Table.2). The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of Denosumab in tablet formulation. The results are furnished in Table 2

Parameters	Values
λ max (nm)	250
Beer's law limit (µg/ml)	2-12
Correlation coefficient	0.999
Retention time	3.64
Theoretical plates	15615
Tailing factor	1.09
Limit of detection ppm	0.15
Limit of quantification ppm	0.5
Slope	2015.607
Intercept	183.357
accuracy	99.58%
R.S.D.	0.837
% of Denosumab in formulation	99..02

Mobile phase	Methanol:water: O.P.A(90:09:01)
P^H	5.2
UV detection	233 nm
Analytical column	C18
Flow rate	1.0ml/min
Temperature	ambient
Injection volume	20µl
Runtime	8 min
Retention time	3.64 min

Table.2 Chromatographic conditions

Recovery

The recovery test was performed at 50%, 100%, 150 % levels. The mean recovery was 98.15%. The results showing our method is accurate.

% Concentration	% Recovery	Mean Recovery
50%	99.63%	
100%	98.82%	98.15%
150%	96.01%	

Table 3: Recovery studies of Denosumab

CONCLUSION

A validated RP-HPLC method has been developed for the determination of Denosumab in tablet dosage form. The proposed method is simple, rapid, accurate, precise and specific. Its chromatographic run time of 8 min allows the analysis of a large number of samples in short period of time. Therefore, it is suitable for the routine analysis of Denosumab in pharmaceutical dosage form.

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