A Validated Method for the Estimation of Casimersen Using RP-HPLC in Bulk and Pharmaceutical Dosage Form

Gowri Kusuma Kumari and Kantipudi Rambabu

Department of Chemistry, RVR & JC College of Engineering, Chowdavaram, Guntur, Andhra Pradesh, India

ABSTRACT

New validated method for the estimation of Casimersen using RP-HPLC In Bulk and Pharmaceutical Dosage form. Chromatographic separation was achieved on a Waters Phenyl Hexyl column (150x4.6mm, 3.5 μ) using isocratic elution with a mobile phase containing buffer and acetonitrile within the ratio of 50:50 as mobile phase with a flow of 1 ml/min at ambient temperature and UV detection was carried at 227 nm. Analysis was achieved within 5 min over an honest linearity within the concentration range from 10-150 μ g/ml of Casimersen. By injecting the standard six times, system suitability parameters were studied and the outcomes were well under the acceptance criteria. Precision and recovery study results were found to be within the suitable limit. By using the above technique assay of marketed formulation was performed and found to be within the limit. Degradation studies were carried out on Casimersen, with a purity threshold greater than purity angle in all conditions and within the acceptable range. The above mentioned technique was validated according to ICH guidelines

KEY WORDS: HPLC, CASIMERSEN, DEVELOPMENT, VALIDATION, DEGRADATION STUDIES.

INTRODUCTION

Casimersen, sold under the brand name Amondys 45, is an antisense oligonucleotide medication used for the treatment of Duchenne muscular dystrophy (DMD) in people who have a confirmed mutation of the dystrophin gene that is amenable to exon 45 skipping. It is an antisense oligonucleotide of phosphorodiamidate morpholino oligomer (PMO). The most common side effects include upper respiratory tract infections, cough, fever, headache, joint pain and throat pain. Casimersen was approved for medical use in the United States in February 2021 and it is the first FDA-approved targeted treatment for people who have a confirmed mutation of the DMD gene that is amenable to skipping exon 45.

MATERIAL AND METHODS / EXPERIMENTAL DETAILS

2.1 Materials: Acetonitrile, Potassium dihydrogen ortho phosphate and ortho phosphoric acid, water (HPLC grade) were purchased from Merck (India) Ltd, Worli, Mumbai, India. All APIs of Casimersen as reference standards were procured from Glenmark Pharmaceuticals., Mumbai.

2.2 Tools: An HPLC system (Waters alliance e2695 model) consisting of quaternary pump, PDA detector-2998 was used. Data processing was performed with Empower 2.0 software.

2.3 Chromatographic Conditions: Chromatographic separation was carried out in isocratic mode at room temperature using Waters Phenyl Hexyl column (150x4.6 mm, 3.5 μ). A mixture of acetonitrile and buffer in 50:50 v/v at a flow of 1 ml/min was used as mobile phase. The injection volume was 10 μ l and the run time was 5.0 min.

2.4 Preparation Of Buffer: Take 1.36gm of Potassium dihydrogen ortho phosphate is dissolved in 1 Lt of HPLC grade water adjust pH-2.5 with OPA and filtered through 0.45 μ filter paper.

2.5 Diluent: Mobile phase was used as diluent.

2.6 Preparation Of Standard And Quality Control Samples: Accurately weigh and transfer 100 mg of Casimersen in 100 ml volumetric flask and add approx. 70 ml

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of diluent, sonicate to dissolve it for 30 min. and made up to the mark with diluent. Further dilute 5 ml of the above solution to 50 ml with diluents.

2.7 Preparation of Sample Solution: Accurately weigh and transfer weight equivalent to 100 mg of Casimersen sample in 100 ml of volumetric flask and add 70 ml of diluent. Sonicate to dissolve and dilute up to the mark with diluent. Take 5 ml of the above solution and diluted to 50 ml and filtered through 0.45 μ nylon syringe filter. From literature survey there is no literature available in Casimersen till today. So that we take and developed new eco friendly method.

2.8 Method Validation

2.8.1 System Suitability: System suitability parameters were measured to verify the system performance. The parameters including USP plate count, USP tailing and % of RSD are calculated and found to be within the limit.

2.8.2 Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of other components, which may be expected to be present in the sample and standard solution. It was checked by examining the chromatograms of blank samples and samples spiked with Casimersen.

2.8.3 Accuracy: Accuracy is the closeness of the test results obtained by the method to the true value. It was assessed by the recovery studies at three different concentration levels. In each level a minimum of three injections were given and amount of the drug present, percentage recovery and related standard deviation were calculated.

2.8.4 Precision: Precision of the analytical method is the degree of agreement among individual test results. It was studied by analysis of multiple sampling of homogeneous sample. The precision of the present method was assessed in terms of repeatability, intraday and inter-day variations. It was checked by analyzing the samples at different time intervals of the same day as well as on different days.

2.8.5 Linearity: Linearity of an analytical method is its ability to obtain results directly proportional to the concentration of the analyte in the sample within a definite range. The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

2.8.6 Stress Degradation: Stress degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. Stress degradation studies were performed as per ICH guidelines Q1 (A) R2. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principle peaks shall pass. Forced degradation studies

were performed by different types of stress conditions to obtain the degradation of about 20%.

2.8.7 Robustness: Robustness of an analytical procedure is a measure of its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness study was performed by injecting standard solution into the HPLC system and altered chromatographic conditions such as flow rate (± 0.2 ml/min), organic content in the mobile phase ($\pm 10\%$). The separation factor, retention time and peak asymmetry were calculated by determining the effect of the modified parameters.

RESULTS AND DISCUSSION

The current study was designed to develop a simple, precise and rapid analytical RP-HPLC method, which can be used for the analysis of assay method for the estimation of Casimersen in bulk and pharmaceutical dosage form. The chromatographic conditions were optimized in order to provide a good performance of the assay. To optimize mobile phase, various combinations were tried for Casimersen. The final working mobile phase is buffer and acetonitrile in the composition of 50:50 v/v. Mobile phase for drug was selected based on its polarity. Detection was carried out in several wavelengths in order to obtain enough sensitivity for the two APIs in smaller proportion (Casimersen). At last the wave length 227 nm was selected shows good absorbance. The flow rate was 1.0 ml/min. The retention time for Casimersen were 3.251 min respectively. The proposed method is validated in accordance with the ICH guidelines with all of the results within the limits. The detection was carried out with a total runtime of 10.0 min. Optimized chromatographic conditions were shown in table 1.

Table 1. Optimized chromatographic conditions

Parameter	Condition
Stationary phase	Waters Phenyl Hexyl (150x4.6 mm, 3.5 μ)
Mobile phase	Buffer: Acetonitrile (50:50, v/v)
Injection volume	10 μ l
Flow rate	1.0 ml/min
Column temperature	Ambient
Wave length	227 nm
Run time	5.0 min
Retention time of Casimersen	3.251 min
* nm: Nanometer	

System Suitability: The system suitability was performed by injecting standard solution containing 100 μ g/ml of Casimersen in six replicates. The results indicate that the system suitability parameter is within the limit. System

suitability results were shown in table 2 and the standard chromatogram was shown in figure 1.

Table 2. Results of system suitability

Parameter	Casimersen
Theoretical plate count	8762
Tailing factor	0.84
Resolution	-
Retention time	3.252

Figure 1: Chromatogram of system suitability

Specificity: There was no interference from blank at the retention time of Casimersen. Figure 2 represents the blank chromatogram.

Figure 2: Chromatogram of blank

Table 3. Results of linearity

S. No	Casimersen Concentration (µg/ml)	Area
1	10.00	259986
2	25.00	649966
3	50.00	1299932
4	100.00	2599863
5	125.00	3249829
6	150.00	3799856

Linearity: Linearity was determined by plotting a calibration curve of peak area against their respective concentration. From this calibration curve it was found that the curve was linear in the range of 10-150 µg/ml of Casimersen. The regression equations for calibration

curve of Casimersen was $Y = 25599x + 11930$ ($R^2 = 0.9998$), the results were shown in table 2 and the calibration plots were shown in figure 5.

Figure 3: Calibration plots of Casimersen

Table 4. Results of method precision & Intermediate precision

S. No.	Area of method precision	Area of Intermediate precision
1	2545634	2566789
2	2555741	2577465
3	2563782	2556644
4	2555578	2598886
5	2557783	2568893
6	2544788	2588937
Mean	2553884	2576269
Std. dev	7352.42	15510.34
% RSD	0.28	0.60

Table 5. Results of accuracy

Accuracy	Amount of Casimersen	% Recovery
50	50	100.04
100	100	100.21
150	150	99.98

Table 6. Results of robustness

Parameter	% RSD of Casimersen
Flow (0.8 ml/min)	0.45
Flow (1.2 ml/min)	0.72
Organic phase (55:45)	1.01
Organic phase (45:55)	0.58

Precision: Precision of this method was assessed in terms of intraday (repeatability) and (intermediate precision) variations. The intraday studies were determined by performing six repeated analysis of the sample solution of Casimersen on the same day under the same

experimental conditions. The intermediate precision of the method was carried out in the same laboratory by studying the analysis with different analyst and different instrument. The method is highly precise as %RSD values were found to be <2%. Good recoveries of the drug were obtained at each added concentration, indicating that the method was accurate. Table 4 gives the method precision and intermediate precision.

Table 7. Results of forced degradation

Stress parameter	% of degradation Casimersen
Acid degradation (1N HCl+ reflux+ 24 hrs)	15.5
Alkali degradation (1N NaOH+ reflux+ 24 hrs)	12.8
Peroxide degradation (30% Peroxide+ reflux+ 24 hrs)	11.6
Photo degradation (UV light (200 W h/m ²) and fluorescent light (1.2 million lux-hrs))	14.3
* nm: Nanometer	

Figure 4: Chromatogram of acid degradation

Figure 5: Chromatogram of alkali degradation

Accuracy: The accuracy of the method was performed by calculating the recovery experiments at three levels (50%, 100% and 150%). APIs with concentration 50, 100, 150 µg/ml of Casimersen were prepared. The test solution was injected three times for each spike level and assay was performed as per the test method. The recovery results were close to 100% and also the RSD values were less than ±2%. The percentage recovery, mean and relative standard deviation were calculated.

Figure 6: Chromatogram of peroxide degradation

Figure 7: Chromatogram of photo degradation

Recovery values demonstrated that the method was accurate within desired range. Accuracy results were shown in table 5.

Robustness: Robustness of the chromatographic method was determined by varying flow rate and mobile phase composition. % RSD was found to be within the acceptable limit. Robustness results were shown in table 6.

Forced Degradation: The proposed method can be used for release and stability studies for effective evaluations and can be considered as stability indicating method. The forced degradation study was carried out according to the ICH requirements include acid, base, oxidation, photo degradation. From the chromatograms it is evident that the selected drugs were stable under the applied stress conditions though the degraded peaks were observed. Forced degradation study results were shown in table 7 and the degradation chromatograms were shown in figure 5, 6, 7 and 8 respectively. However, the study of forced degradation had never been done or published in any journal, it was the first stabilising LC technique for Casimersen quality control. The devised approach was validated in accordance with ICH requirements and found to be very precise, quick, simple, cost-effective, and sensitive to the specific pharmaceutical dosage form.

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Authors' Contributions: Autor 1 designed the study, performed the statistical analysis, wrote the protocol, and wrote the manuscript. Autor 2 to check the work and review the manuscript.

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