METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF VENETOCLAX AND OBINUTUZUMAB BY USING NP-HPLC IN ACTIVE PHARMACEUTICAL INGREDIENT FORM

Dr. M. DAVID RAJU

Department of Chemistry, PB Siddhartha College of Arts and Science, Vijayawada, AP.

E.mail ID: davidm201926@gmail.com

Abstract

The aim of this study is to create and validate a fast, easy-to-use, affordable, sensitive, and accurate method for measuring Venetoclax and Obinutuzumab in bulk and pharmaceutical products using Normal-Phase High-Performance Liquid Chromatography (NP-HPLC). Hypersil chiral column was used with a running phase composed of Methanol: n-Hexane: 0.1% Formic acid (60:20:20 v/v) at a flow rate of 1.0 ml/min. UV detection was used at a wavelength of 225 nm. Obinutuzumab and Venetoclax correlation coefficients were found to be 0.999 over a concentration range of 6.25-37.5 µg/ml and 2.5-15 µg/ml, respectively. Venetoclax and Obinutuzumab have respective retention times of 3.134 and 6.929 minutes. The run time for separating Obinutuzumab and Venetoclax peaks was 10 minutes. This method, proposed as a regular analysis and quality control tool for medications that contain these active drugs either individually or in combinatio, was evident to be a suitable one.

Keywords: *HPLC, Obinutuzumab, Venetoclax, Development, Validation.*

1. Introduction

Venetoclax, sold under the brand names Venclexta and Venclyxto, is a medication used to treat adults with chronic lymphocytic leukemia (CLL)[1-2], small lymphocytic lymphoma (SLL), or acute myeloid leukemia (AML)[3-4]. The most common side effects are low levels of neutrophils[5] (a type of white blood cell), diarrhea[6], nausea, anemia (low red blood cell counts), nose and throat infection[7] and tiredness. Venetoclax attaches to a protein called Bcl-2[8-9]. This protein is present in high amounts in CLL cancer cells, where it helps the cells survive for longer in the body and makes them resistant to cancer medicines. By attaching to Bcl-2 and blocking its actions, venetoclax causes the death of cancer cells and thereby slows down progression of the disease.

Obinutuzumab, sold under the brand name Gazyva among others, is a humanized anti-CD20 monoclonal antibody[10-11], originated by GlycArt Biotechnology AG and developed by Roche as a cancer treatment. It can be used as a first-line treatment for chronic lymphocytic leukemia in combination with chemotherapy[12-13] or with venetoclax, as a first-line treatment for follicular lymphoma[14-15] in combination with chemotherapy, and as treatment for relapsed or refractory follicular lymphoma in combination with bendamustine[16] chemotherapy. Obinutuzumab is used in combination with chlorambucil[17] as a first-line treatment for chronic lymphocytic leukemia.

2. Experimental Study

Solutions and Reagents

The pure Obinutuzumab and Venetoclax used in this study was provided by Glenmark Pharmaceutical Private Ltd., located in Andheri (E), Mumbai, India (99.8-99.9 percent purity). Other reagents, including methanol, formic acid, n-hexane and water, were obtained from Merck (India) Ltd. in Worli, Mumbai, India, and were of HPLC grade.

Collection of instruments

Waters alliance liquid chromatography (model 2695) monitored with empower 2.0 data handling system and a detector of photo diode array (model 2998) was used for this study.

Step of mobility

For Standard review, the mobile step was methanol: n-Hexane: Formic acid (60:20:20 v/v) and was degassed beforehand. A mobile phase chosen to produce well-defined peaks with a low tailing factor (2.0) and a plate count of over 2000 was selected.

Prepare the diluent

Mobile phase was used as diluent.

Conditions of Chromatography

For the HPLC experiments, Hypersil Chiral cell column (150 x 4.6 mm, 3 µm) was used. The elution was conducted with isocratic conditions using methanol: n-Hexane: Formic acid (60:20:20 v/v) at a flow rate of 1.0 ml/min. The injection volume was 10 µl, and the run time was 10 minutes, with the column temperature set to room temperature and the absorbance measured at 225 nm (Because maximum absorbance was observed at this wavelength. So, this was selected as wavelength).

Standard Solution Preparation

To dilute 25 mg of Obinutuzumab and 10 mg of Venetoclax, measure out the drug and transfer it to a 100 mL volumetric flask, then add approximately 70 mL of a diluent and sonicate for 30 minutes to dissolve it, after that add more diluent to the total amount. Further dilute 5 mL to 50 mL with diluents.

Validation Process[18-26]

The analytical parameters such as system suitability, precision, specificity, accuracy, linearity, robustness, LOD, LOQ, forced degradation and stability were validated according to ICH Q2 (R1) guidelines.

System Precision

The system's performance has been validated through assessment of device suitability parameters. Limits were found to be met for a variety of parameters, including plate count, tailing, and RSD percentage.

Specificity

Being able to identify and test a given analyte in the presence of other elements required to be combined in the Standard and the standard solution is known as specificity. Blank Standards and those with Obinutuzumab and Venetoclax will be tested using chromatograms.

Accuracy

Being close to the real meaning of the technique is what defines accuracy. Three concentrations will be used to test the recovery trials. The drug's quantity, percentage of recovery, and standard deviations were calculated after every injection at each level.

Precision

It is the level of agreement between the various test results that determines the precision of the analytical methodology. Researchers examined the effects of sampling a homogeneous population more than once. The current process was evaluated in terms of

its ability to provide repeatable, intraday, and inter-day results. It was examined by sampling the materials on the same day and over the course of different days.

Linearity

Linearity is the feature of analytical process which allows for a direct proportion of analytical results in response to a certain concentration of the analyte in the Standard. A total of seven series of standard solutions were selected for the assessment of the linearity spectrum. The calibration curve was drawn by comparing regular solution concentration with peak area. Using the least square method, the slope, intercept, and coefficient of correlation were calculated.

Forced Degradation

The peaks in the chromatogram should agree. ICH guidance Q1 (A) R2 was performed in conjunction with stress degradation experiments. The peaks of degradation should be well distanced and at least 1.0 resolution between peaks. For the largest peaks to go over, a separation must occur. A degradation of around 20 percent has been attained via several various stress conditions like acid, alkali, peroxide, reduction, thermal and photo in what is known as a forced degradation experiment.

Robustness

Robustness refers to a procedure's resistance to small process parameter changes, as well as its reliability in normal operation. An organic solution was introduced into the HPLC system for a robustness analysis, and the chromatographic settings (such as flow rate and mobile-phase organic content) were modified. The separation factor, retention time, and peak asymmetry were determined by evaluating the effects of altered parameters.

3. Results and Discussion

The aim of this study is to establish a single isocratic HPLC method for the simultaneous quantification of Obinutuzumab and Venetoclax in bulk and pharmaceutical dosage forms that is reliable, precise, and cost effective. According to the UV spectra of these compounds, an appropriate wavelength for simultaneous estimation of two drugs was chosen.

Optimization of the method

The separation was achieved using Hypersil chiral cell column (150mm x 4.6mm, 3µm) and a mobile phase of methanol: n-Hexane: Formic acid (60:20:20 v/v) with a flow rate of 1.0 ml/min and UV detection at a wavelength of 225 nm. The entire performance lasted six minutes. Conditions for optimized chromatography are provided in table 1.

System Suitability

To attain results, the following device suitability parameters were established after six consecutive injections of normal solution: theoretical plate number, time, peak area, tailing factor, and resolution. The chromatogram in Figure 1 was the representative of the suitability results detailed in table 2.

Table 1. Method suitability conditions

Parameter	Suitable conditions
Column	Hypersil chiral cell column (150 x 4.6 mm, 3 µ)
Moving Phase	methanol: n-Hexane: Formic acid (60:20:20 v/v)
Volume of injection	10 µl
Stream rate	1.0 mL/min
Temperature of column	25°C
Wavelength	225 nm
Time duration	10 minutes

Table 2. Results of system suitability

Parameter	Venetoclax	Obinutuzumab
Number of plates	4658	12147
Tailing	1.15	1.02
Resolution		17.45
Peak elution time	3.134	6.929
% RSD	0.87	0.95

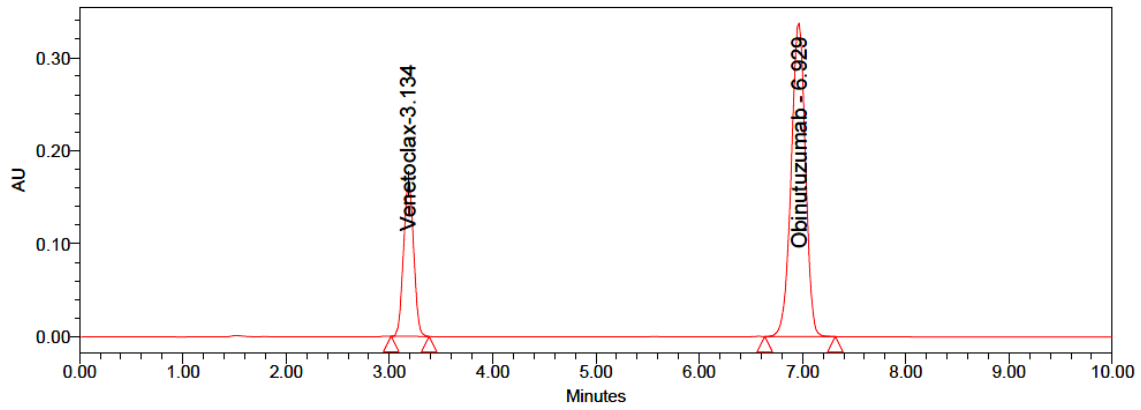

Figure 1. Chromatogram of standard

Specificity

There was no participation from Obinutuzumab and Venetoclax at the elution time. As seen in Figure 2, the blank chromatogram is present.

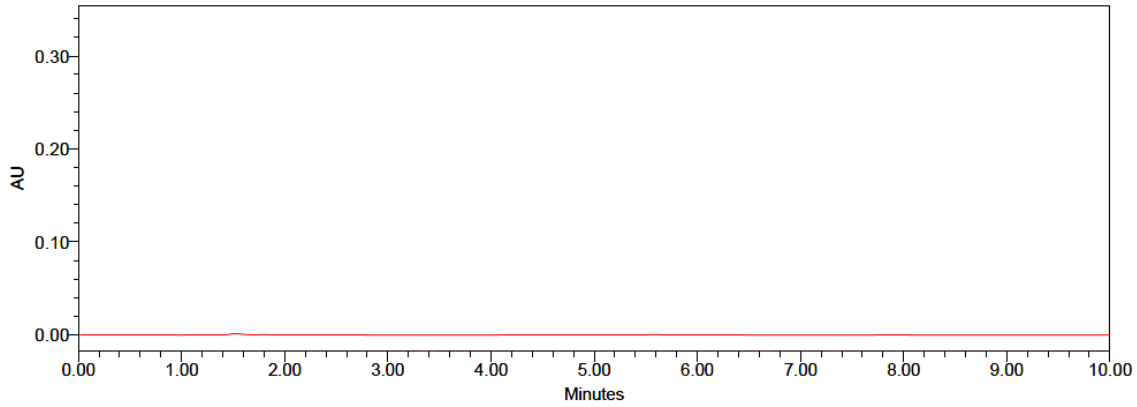

Figure 2. Chromatogram of blank

Linearity

By using a calibration curve to determine the linearity of the area of peak, its corresponding concentration was discovered. From this graph, it appears that the range of 2.5-15 µg/mL of Venetoclax and 6.25-37.5 µg/mL of Obinutuzumab had a straight line. Linearity results were demonstrated in table 3.

Table 3. Results of linearity

S. No	Venetoclax		Obinutuzumab	
	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area
1	2.50	312624	6.25	807383

2	5.00	606984	12.50	1634638
3	7.50	833647	18.75	2464572
4	10.00	1168351	25.00	3230368
5	12.50	1472646	31.25	4150920
6	15.00	1751942	37.50	4787679

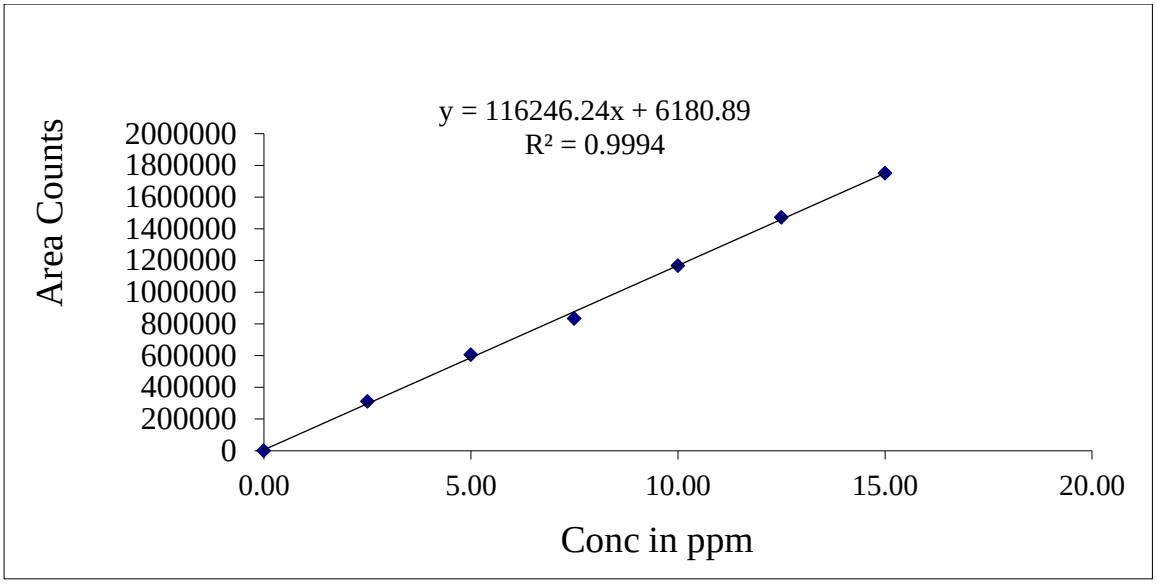

Figure 3. Calibration plot of Venetoclax

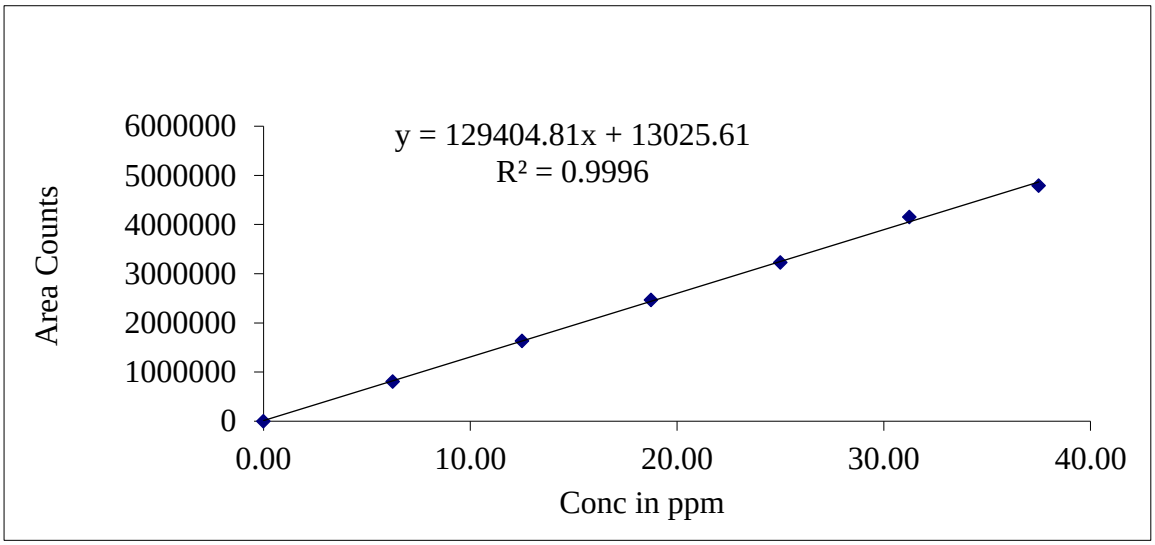

Figure 4. Calibration plot of Obinutuzumab

Precision

Intraday and intermediate precision variances were assessed in relation to the procedure's accuracy. The samples were examined six times on the same day to obtain intraday results for Obinutuzumab and Venetoclax. The system's intermediate precision was explored by analyzing data in the same laboratory using a variety of examiners and tools. It is very accurate, with an RSD percentage of less than 2%. The process was precise,

yielding the best drug recoveries at each additional concentration. Table 4 shows the method precision results.

Table 4. Outcomes of method precision

S. No.	Venetoclax		Obinutuzumab	
	Area	% Assay	Area	% Assay
1	1170571	100.5	2725648	99.4
2	1144840	98.2	2701256	98.5
3	1178354	101.1	2735642	99.7
4	1164361	99.9	2748143	100.2
5	1183214	101.5	2772874	101.1
6	1156521	99.2	2754123	100.4
Mean	1166310	100.1	2739614	99.9
Std. dev	14209.46	1.231	24785.66	0.898
% RSD	1.218	1.23	0.905	0.9

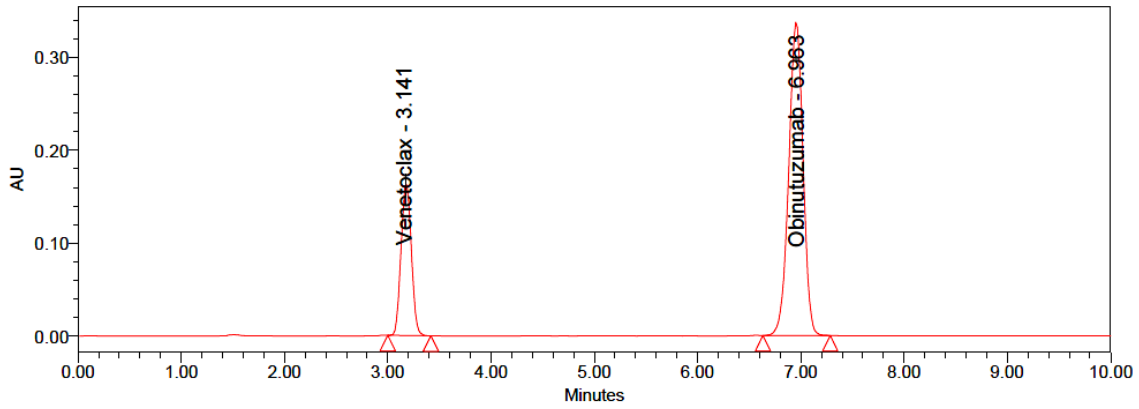

Figure 5. Chromatogram of method precision

Intermediate Precision (Ruggedness)

Intermediate precision results were shown in table 5.

Table 5. Results of intermediate precision

S.No.	Venetoclax		Obinutuzumab	
	Area	% Assay	Area	% Assay
1	1142123	98.8	2727654	99.4
2	1190154	101.9	2703216	98.5
3	1157854	99.2	2746241	100.1
4	1173215	100.6	2767548	100.9
5	1168974	100.2	2758452	100.5
6	1188820	101.9	2784543	101.5
Mean	1170190	100.4	2747942	100.2
Std dev	18418.47	1.309	29152.7	1.077
% RSD	1.574	1.3	1.061	1.07

Accuracy

By measuring the recovery experiments at three stages, the method's precision was reached (50 percent, 100 percent, and 150 percent). APIs were made with concentrations of Obinutuzumab of 5, 10, and 15 micrograms/mL and Venetoclax of 12.5, 25 and 37.5 micrograms/mL. For each stage of the spike, the test solution was injected three times, and the assay was performed in accordance with the test process. In addition to being

able to determine the percentage of recovered data, the mean and relative standard deviations have also been found. The strategy was effective because the recovery values fell within the target range. Table 6 presents the accuracy results.

Table 6.Results of accuracy

Accuracy	Amount of Venetoclax	% Recovery	Amount of Obinutuzumab	% Recovery
50*****	5	101.4	12.5	100.3
100*	10	100.2	25	99.9
150*	15	100.2	37.5	98.7

* Results are mean recovery of three sample preparations

LOD and LOQ

The concentration level at which the analytes are reliably detected and quantified is the limit of detection and quantification. Venetoclax and Obinutuzumab had a LOD concentrations of 0.03 µg/ml, 0.075 µg/ml and their S/N values of 3, 3. The LOQ concentrations of Venetoclax and Obinutuzumab were 0.1 µg/ml, 0.25 µg/ml, and their S/N values were 10, 10. (S/N is the ratio of signal to noise).

Robustness

To ensure the robustness of the chromatographic technique, the researchers evaluated flow rate and the composition of the mobile phase. By changing the flow rate and mobile phase ratio, the area of drugs changes. So, the percentage of relative standard deviation changes. Here in Table 7 (robustness results) the %RSD values are in within the acceptable limit.

Table 7. Outcomes of robustness

Parameter	% RSD of Venetoclax	% RSD of Obinutuzumab
Flow (0.8 mL/min)	0.65	1.37
Flow (1.2 mL/min)	0.86	0.6
Organic phase (66:17:17)	0.71	0.69
Organic phase (54:23:23)	0.85	1.42

Forced Degradation

The proposed approach can be used for successful evaluations of release and stability tests, and it can be called a stability preferable technique. Acid, Alkali, oxidation, reduction, photo, and thermal degradation are all included in the ICH-required forced degradation analysis. The chromatograms show that the selected drugs remained stable under the stress conditions, despite the presence of degraded peaks. Results of forced degradation were given in table 8.

Acid degradation

A volume of 1 ml standard stock solution was transferred to a volumetric flask with a capacity of 10 ml, to which 1 ml of 1N HCl was added and left to stand for 15 minutes. After 15 min add 1 ml of 1N NaOH and make up to the diluent mark. Filter the solution using syringe filter and injected into HPLC system.

Alkali degradation

A volume of 1 ml standard stock solution was transferred to a volumetric flask with a capacity of 10 ml, 1 ml of 1N NaOH was added, and the mixture was left to stand for 15 minutes. After 15 minutes, add 1 mL of 1N HCl to bring the solution up to the required concentration. Use a syringe filter to filter the solution, which will then be injected into the HPLC system.

Peroxide degradation

A volume of 1 ml standard stock solution was moved to a volumetric flask of 10 ml, add 1 ml of 30% hydrogen peroxide solution and make up to the mark with diluents. Filter the solution using syringe filter and injected into HPLC system.

Reduction degradation

Using a volumetric flask with a capacity of 10 ml, transfer 1 ml of standard stock solution and add 1 ml of 30% hydrogen peroxide solution, then dilute to the required concentration with diluents. Use a syringe filter to filter the solution, which will then be injected into the HPLC system.

Thermal degradation

During the 6-hour baking period, the standard solution was kept at 105°C. The resulting solution was injected into an high-performance liquid chromatography system.

Photolytic degradation

A weight of 100mg standard was exposed to sunlight for 6 hrs. and the exposed sample was analyzed. Prepare the sample solution by using this sample and inject into HPLC system.

Table 8. Forced degradation results

Stress Parameter (24 hrs)	% Degradation	
	Venetoclax	Obinutuzumab
Acid degradation (1N HCl)	11.4	10.9
Alkali degradation (1N NaOH)	10.6	10.4
Peroxide degradation (30% Peroxide)	13.5	12.4
Reduction degradation (30% sodium bi sulphate)	6.4	5.9
Thermal (sample, 70°C, 6 Hrs)	0.5	1.2
Photo (UV-Vis light- (200 W h/m^2) and fluorescent light (1.2 milliion lux-h)	1.1	1.7

4. Conclusion

In this study, a novel, quick, sensitive, and easy-to-use HPLC method was developed for the simultaneous estimation of Venetoclax and Obinutuzumab in API and pharmaceutical dosage types. Shorter run time, low cost, and all the other characteristics are benefits. All the parameters were verified and were found to be within the acceptable range, including linearity, accuracy, specificity, robustness, and process precision. According to our research, the RSD values for all the parameters came in at less than 2%, showing that the procedure is accurate and that the results we found are consistent. Therefore, it's possible to use the current approach in QC laboratories for routine study and manufacturing Obinutuzumab and Venetoclax pharmaceuticals without having to separate the substances first.

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