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TWO NOVEL METHODS DEVELOPED FOR THE ESTIMATION OF BARICITINIB USING BM-REAGENT AND FLUOROMETRY

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ABSTRACT

Alopecia areata and moderate to severe rheumatoid arthritis can both be treated with the new medication Baricitinib. In addition, it received FDA clearance in 2022 as the first immunomodulatory medication for the management of COVID-19 in hospitalised individuals who needed supplemental oxygen. A selective JAK inhibitor, it inhibits Janus Kinase 1 and Janus Kinase 2 reversibly. Baricitinib in bulk and formulation can be estimated using rapid, accurate, and affordable approaches. The reaction with the BM reagent and methanol as the diluent served as the basis for the development of the visible spectroscopic approach. 10 mg of the drug were dissolved in 1 mL of DMSO (dimethyl sulfoxide) to create the standard stock solution for the Visible spectroscopic technique, which was then made up with methanol. Methanol was used to make the succeeding dilutions, and the maximum wavelength was discovered to be 580 nm. The linearity was discovered to be between 10µg/mL-500µg/mL, with an excellent correlation value (r^2) of 0.998. In the second method that is fluorometric method, Baricitinib standard drug solution and sample tablet solution was prepared by first dissolving the 10mg drug in 0.1N H_2SO_4 and then making it up using double distilled water. The different concentrations of pure

drug in the range 2-16µg/ml and one sample solution were measured for the intensity in the fluorometer. The calibration curve was plotted and the sample's unknown concentration was calculated from the plot. The calibration curve was found to be linear with r^2 value obtained as 0.99. The technique was validated in accordance with ICH Q2 R (2) standards. The accuracy (%RSD 2.0) of the visible method was within acceptable limits. As a result, the established visual approach is sensitive, repeatable, and accurate.

Keywords: Baricitinib, COVID-19, BM reagent, Visible spectroscopic method, fluorometric method

INTRODUCTION

Baricitinib:

Eli/Lilly Company developed Baricitinib [Olumiant®] as a selective, reversible Janus kinase inhibitor to treat arthritis and dermatitis **[1]**. IUPAC name of Baricitinib is 2-[1-[Ethylsulfonyl]-3-[4-7H-pyrrolo[2,3-d]pyrimidin-4-yl-1H-pyrazol-1-yl]azetidin-3-yl] acetonitrile which is an immunomodulatory drug with anti-inflammatory properties. **Figure 1** shows the structure of Baricitinib. For individuals with severe or moderate active rheumatoid arthritis, Baricitinib was authorized for use in the EU in February 2017. It is used as a monotherapy or with methotrexate to treat patients with moderately to severely active rheumatoid arthritis who have not reacted favourably to or are intolerant to one or more disease-modifying anti-rheumatic medications. Its use in combination with REMDS to treat hospitalised patients has been authorised by the FDA in the United States. Following administration, Baricitinib binds to JAK1/2 to stop it from being active, which stops the STAT signalling pathway and JAK signal transducers from being activated as well. As a result, fewer inflammatory cytokines are produced, perhaps delaying the onset of inflammation. Baricitinib may also cause apoptosis and lessen the growth of tumour cells that express JAK1/2.

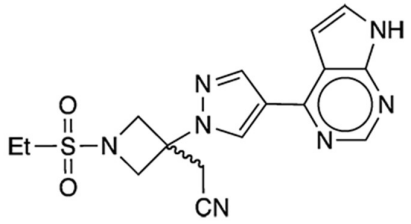

Figure 1: Structure of Baricitinib

BM reagent:

BM reagent is chemically known as N-1-naphthyl ethylene diamine dihydrochloride. The structure is as shown in **Figure 2**. It is a widely used and incredibly sensitive chromogenic reagent for the detection of medicines and medications that contain free primary aromatic amino groups **[2-4]**.

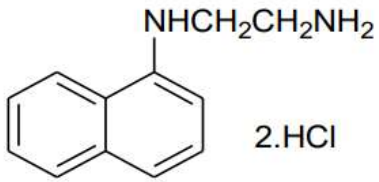

Figure 2: Structure of BM reagent

Principle: Sodium nitrite and hydrochloric acid are used to first diazotize the primary aromatic amino group. The use of an ammonium sulfamate reagent to treat the excess nitrous acid neutralises it. Finally, the diazonium ion can couple with the Bratton-Marshall reagent to create an azodye complex that has a colour.

The literature survey reveals that there are few methods available like the two LC test techniques for determining its pharmacokinetics in rat plasma **[5]**. The former employed LC/MS/MS to estimate Baricitinib and Methotrexate, whereas the latter used UPLC. One UV-spectroscopic method for determining the drug's pure form and dosage form was developed which used DMSO as the diluent. Furthermore, very few HPLC methods are available for the determination of Baricitinib like the one which uses RPLC-Diode array detection system, the second method used Methanol: Phosphate buffer (45:55) as mobile phase with UV detector and other employed QbD approach to in the method development.

Since Baricitinib has a primary aromatic amino group in its structure, the BM reagent can be used to form an azodye complex which produces a colour. The chromogenic method developed using DMSO and methanol diluent and the fluorometric method developed using 0.1N H_2SO_4 diluent and double distilled water **[6]**. These two methods can be used to quantify Baricitinib in bulk and in formulation which is different from routine quantification using the UV spectroscopic method. From the literature search, it was found that there is no visible or chromogenic method and fluorometric method developed for the estimation of Baricitinib and hence there was a need felt to develop these methods which are easy, rapid, and also economical for the estimation of Baricitinib in bulk and in the formulation.

MATERIALS AND METHODS

Chemicals: Baricitinib API (Active Pharmaceutical Ingredient) was a gift from the pharmaceutical industry **[7-8]**. A tablet formulation containing Baricitinib 4mg was bought from the local pharmacy. Dimethyl Sulfoxide (DMSO), methanol, 0.1N H_2SO_4, double distilled water solvents were used.

Instrument: A double-beam UV-Visible spectrophotometer- ELICO 210, Fluorometer ELICO-CL53 was used in the method development and validation.

VISIBLE METHOD USING BM REAGENT:

Selection of solvent: Baricitinib drug was checked for solubility in organic solvents DMSO, DMF, and Methanol **[9]**. DMSO was selected as the solvent to dissolve the drug and methanol was used to make up the dilution and prepare subsequent dilutions during the method development.

Preparation of solutions:

5N HCl: This was prepared by taking 10.42mL of Concentrated Hydrochloric acid in 25mL of water.

Sodium Nitrite Solution (0.3% w/v): This solution was prepared by dissolving 0.3 g of Sodium Nitrite in 100 mL distilled water **[10-11]**.

Ammonium Sulfamate Solution (0.5% w/v): 0.5 % (w/v) ammonium sulfamate is prepared by dissolving 0.5g in 100 mL distilled water.

Preparation of BM Reagent: 100 mg of N-1-naphthyl ethylene diamine dihydrochloride was taken in 100 ml of a mixture of seven parts of acetone and three parts of water.

Preparation of Stock solution:

Stock solution (1000 µg/ml) was prepared by first dissolving 10mg of Baricitinib pure drug in 1ml of DMSO and then made up to mark in the 10ml Volumetric flask with methanol **[12]**. The working standard solution having a concentration 100µg/ml and the subsequent dilutions of required concentrations were prepared using methanol as the diluent.

Procedure:

Baricitinib standard solutions in methanol with concentrations between 10 and 500 µg/mL were poured into a series of 10 mL volumetric flasks. 1.0 mL of 5N hydrochloric acid and 2.0 mL of 0.3% (w/v) Sodium nitrite solution were added to each flask having 2 mL of drug solution, and the flasks were firmly shaken for 2 minutes **[13-14]**. Following the addition of 1.0 mL of 0.5% (w/v) ammonium sulfamate and 2 mL of BM reagent to each flask, the absorbance was measured using a double-beam UV-visible spectrophotometer in the 400-800nm wavelength range. Figure 3 illustrates the sharp peak obtained at 580 nm which showed the maximum absorbance of the drug. The calibration curve was plotted with absorbances of the solutions against the various concentrations **[15]**.

Quantification of Sample:

The 10 tablets containing Baricitinib 4mg were weighed & average weight was calculated. Tablets were powdered using a motor and pestle and the equivalent weight of 10mg pure drug was calculated from the average weight **[16-17]**. The equivalent weight powder was taken in a 10ml volumetric flask and 1ml DMF was added to dissolve it & was then made up with methanol. The procedure as mentioned for the standard solutions was followed for the sample to get a coloured solution after the addition of BM reagent. The actual concentration was calculated from the calibration curve of the standard solution.

FLUOROMETRIC METHOD:

Preparation of solutions:

0.1N H_2SO_4 solution preparation: 0.3ml of concentrated H_2SO_4 was dissolved in small quantity of double distilled water first and

later filled up to the mark using the double distilled water to make the 0.1N H_2SO_4.

Preparation of Stock solution:

Stock solution (1000µg/ml) was prepared by first dissolving 10mg of Baricitinib pure drug in 1ml of 0.1N H_2SO_4 and then made up to mark in the 10ml Volumetric flask with double distilled water **[18]**. The working standard solution having a concentration 100µg/ml and the subsequent dilutions of required concentrations were prepared using double distilled water as the diluent.

Procedure:

Baricitinib standard solutions in double distilled with concentrations between 2-16µg/ml was prepared into a series of 10 ml volumetric flasks **[19]**. The intensity was measured using the fluorometer. The calibration curve was plotted with intensities of the solutions against the various concentrations.

Quantification of Sample:

The 10 tablets containing Baricitinib 4mg were weighed & average weight was calculated. Tablets were powdered using a motor and pestle and the equivalent weight of 10mg pure drug was calculated from the average weight. The equivalent weight powder was taken in a 10ml volumetric flask and 1ml 0.1N H_2SO_4 was added to dissolve it & was then made up with double distilled water **[20]**. The procedure as mentioned for the standard solutions was followed for the sample. The actual concentration was calculated from the calibration curve of the standard solution.

METHOD VALIDATION OF VISIBLE METHOD:

Linearity and Range: Linearity of Baricitinib was determined from the calibration curve. The correlation coefficient (r^2) along with the equation (y= mx+c) was obtained by linear regression analysis. The range in which the drug shows linear response was also noted.

Precision: Repeatability of method was determined by analysing replicates of same level of concentration 6 times i.e., at 100 µg/ml. The inter-day and intra-day precision estimated by repeating the analysis of 100 µg/ml on different days by the same analyst and on the same day by different analysts respectively for the determination of the Intermediate precision. The precision is calculated by calculating the %RSD.

Accuracy: The accuracy of the method was confirmed by spiking the sample concentration with 50%, 100%, and 150% of standard drug concentration i.e., to the sample concentration of 50 µg/ml, 25 µg/ml, 50 µg/ml, and 75 µg/ml of Baricitinib standard concentration was added and the recovery percentage was calculated. The spiking was done three times and the mean percentage recovery was then calculated.

Detection limit and the Quantitation limit: Detection limit and the quantitation limit of the method developed were

calculated from the calibration standards. The detection limit was calculated from the formula as per ICH guidelines Q2 R (2).

$$DL = \frac{3.3\,\sigma}{S}$$

$$QL = \frac{10\,\sigma}{S}$$

Where σ = Standard deviation of the response.

S = Slope of the calibration curve.

Robustness: The method developed was also validated with the robustness parameter. A small change in the method developed is assessed. Here the 100µg/ml solution of the drug was scanned at + & - 1 nm of the λmax 580nm.

CALCULATIONS:

Assay calculations:

The percentage purity of the marketed sample is calculated using the formula.

$$\%Assay = \frac{Absorbance\ of\ Sample}{Absorbance\ of\ Standard}\ x\ \frac{Concentration\ of\ Standard}{Concentration\ of\ Sample}\ x\ 100$$

$$\%\text{Assay} = \frac{0.4612}{0.4521}\ \text{x}\ \frac{50}{51.83}\ \text{x}\ 100$$

% Assay = 98%

RESULTS AND DISCUSSION:

The prepared dilutions of the pure Baricitinib were scanned in the visible range of 400-800nm. A sharp peak was obtained at λmax 580nm as seen in **Figure 3**. The drug obeyed the Beer-Lambert's law in the calibration curve range of 10-500 µg/ml where the linear response of the drug was recorded. Correlation coefficient was found to be 0.998 with the equation y = 0.0056x + 0.1738 as shown in the graph in **Figure 4**. The method developed showed good repeatability with % RSD = 0.6024 % which is shown in **Table 1**. Intermediate precision with Intra-day and the Inter-day precision calculations were also performed and the results obtained had % RSD < 2.0 as mentioned in **Table 2& 3** respectively. The recovery percentages were calculated for the accuracy of the method developed. The data related to the spiked studies for accuracy is shown in Table 4 and the mean % recovery of the drug was calculated to be in the range 97-99%. The detection limit and the quantitation limit calculated from the equations as mentioned in ICH Q2 R (2) guidelines were found to be 2.722 µg/mL and 8.251 µg/mL respectively. The method developed was robust with the % RSD = 0.2572 % and 0.3569 % at 579 nm and 581 nm respectively as mentioned in **Table 5**.

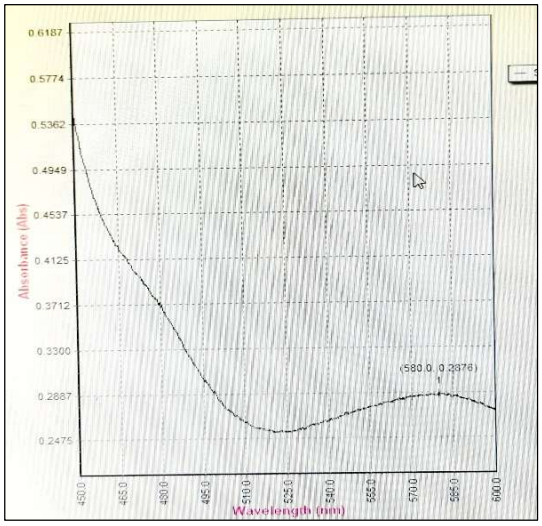

Figure 3: Determination of λmax of Baricitinib pure drug

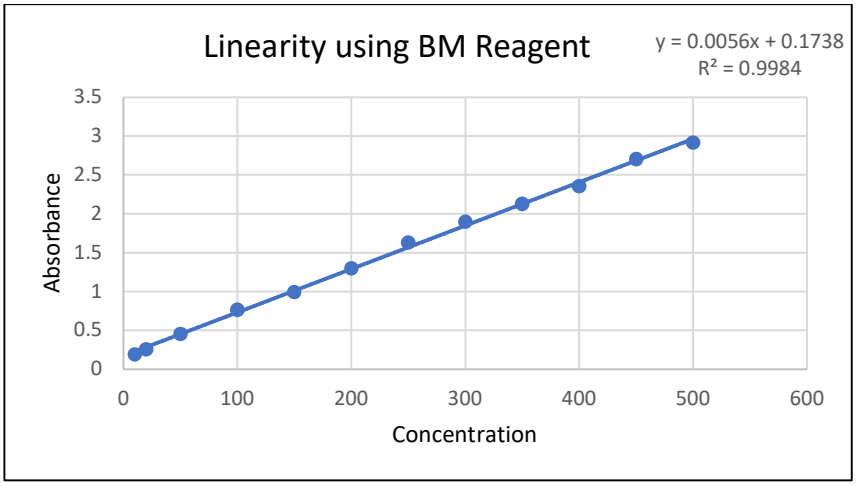

Figure 4: Linearity of Baricitinib using BM reagent

Table 1: Repeatability of 40 µg/ml Baricitinib drug solution [21]

Number of repetitions	Absorbance
1	0.7653
2	0.7681
3	0.7654
4	0.7712
5	0.7597
6	0.7724
Average	0.767016667
Standard Deviation	0.00462057
% Relative standard deviation	0.602408025

Table 2: Intra-day precision data of 40 µg/ml Baricitinib drug solution

Number of repetitions	Analyst-1	Analyst-2
1	0.7653	0.7196
2	0.7681	0.7212
3	0.7654	0.7234
4	0.7712	0.7188
5	0.7597	0.7179
6	0.7724	0.7184
Average	0.767016667	0.719883333
Standard Deviation	0.00462057	0.002073081
% Relative standard deviation	0.602408025	0.287974641

Table 3: Inter-day precision data of 40 µg/ml Baricitinib drug solution [22]

Number of repetitions	Day-1	Day-2
1	0.7653	0.7534
2	0.7681	0.7512
3	0.7654	0.7538
4	0.7712	0.7515
5	0.7597	0.7543
6	0.7724	0.7576
Average	0.767016667	0.753633333
Standard Deviation	0.002115879	0.002312286
% Relative standard deviation	0.356939711	0.306818439

Table 4: Recovery studies data of the Method developed

Percentage level	Absorbance	% Recovery	Mean % Recovery
50% (50ppm+25ppm)	0.7213	98.60%	
	0.7217	98.66%	
	0.7222	98.72%	98.66%
100% (50ppm+50ppm)	0.8932	99.01%	
	0.8935	99.04%	
	0.8931	99.00%	99.02%
150% (50ppm+75ppm)	0.9445	97.24%	
	0.9449	97.28%	
	0.9439	97.17%	97.23%

Table 5: Robustness data of the Method developed

Number of repetitions	Absorbance at 579 nm	Absorbance at 580 nm	Absorbance at 581 nm
1	0.6512	0.7653	0.5987
2	0.6497	0.7681	0.5932
3	0.6547	0.7654	0.5993
4	0.6539	0.7712	0.5894
5	0.6572	0.7597	0.5885
6	0.6611	0.7724	0.5876
Average	0.654633333	0.767016667	0.592783333
Standard Deviation	0.001683977	0.00462057	0.002115879
% RSD	0.257239693	0.602408025	0.356939711

The prepared dilutions of the pure Baricitinib were scanned for the intensities with different concentrations. The calibration curve was plotted using the intensities of the different concentrations in the range 2-16µg/ml and the Correlation coefficient was found to be 0.99 with the equation y = 7.0274x+ 0.6286 as shown in the graph in Figure 5. Table-6 shows the intensities of the concentrations **[23]**.

Table 6: Intensities of the Baricitinib different concentrations.

Concentration	Intensity
2	14.6
4	27
6	43.2
8	57.2
10	74.1
12	84.8
14	97.3
16	112.8

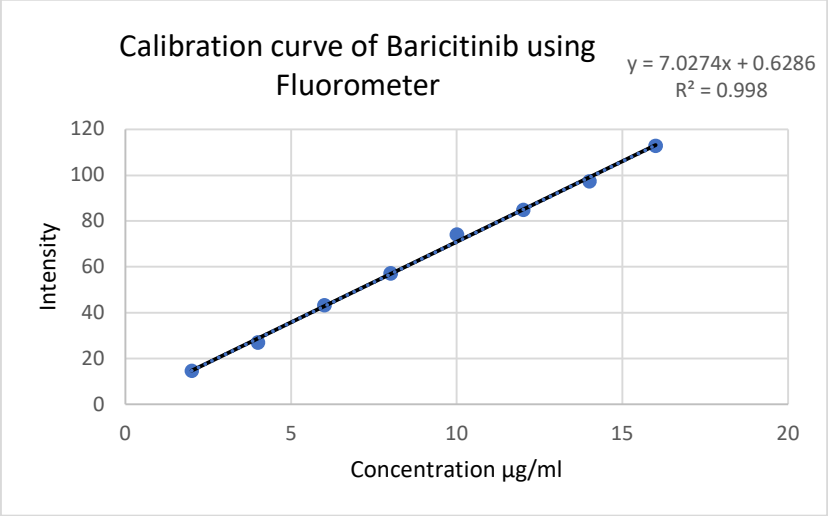

Figure 5: Linearity of Baricitinib using Fluorometer

CONCLUSION:

The measurement and quantification of Baricitinib using BM reagent in bulk and formulation was found to be simple, accurate, linear, robust, and quick using the suggested UV-visible spectroscopic approach. The fluorometric method developed was also simple and rapid. This chromogenic technique developed is simple and produces accurate results. Since both the methods are practical and affordable, it may be used for regular quality control examinations of the Baricitinib dosage form.

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