Analytical method development and validation for simultaneous estimation of API in pharmaceutical dosage form by using RP-HPLC method.

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***Abstract*—A simple, specific and sensitive reverse phase high performance liquid chromatographic method was developed and validated for simultaneous determination of vonoprazan and amoxicillin from pharmaceutical dosage forms. The method uses Hypersil, C18 (250mm x 4.6ID, Particle size: 5 um) column and isocratic elution. The mobile phase composed of MeOH:Water:Glacial acetic acid, at the ratio of 75:24.5:0.5 v/v was used at a flow rate of 1.0 ml /min. UV detector was programmed at 232 nm for first 10 min and at 244 nm for 10 to 20 min. All the validation parameters were in acceptable range. The developed method was effectively applied to quantitate amount of vonoprazan and amoxicillin from tablets. The method was also applied suitably for determining the degradation products of vonoprazan and amoxicillin .**

Keywords: Vonoprazan, reverse phase high performance liquid chromatography, amoxicillin, analysis, tablets

I. INTRODUCTION

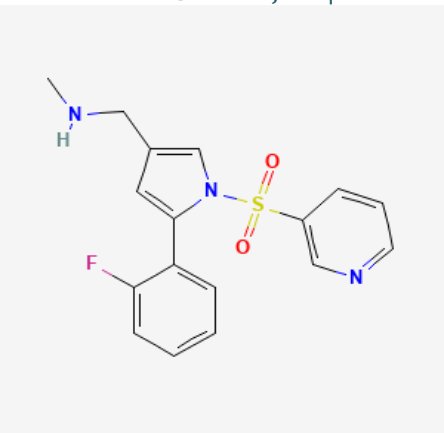
High-Performance Liquid Chromatography (HPLC) evolved from traditional column chromatography and is now one of the most essential tools in analytical chemistry. In the modern pharmaceutical industry, HPLC plays a crucial role at every stage of drug discovery, development, and production.

(H. pylori) is a bacteria responsible for one of the most widespread and persistent infections in the world. Nearly half of the global population is affected by it. In developing countries, the infection rate can be as high as 90%, while in developed nations (except Japan), it remains below 40%.

There are several ways to diagnose an H. pylori infection, and the choice of method depends on factors like test availability, cost, accessibility, whether an endoscopy is required, and the patient's age.[9]

When it comes to treatment, the standard approach is *triple therapy*, which typically involves a combination of antibiotics and acid-reducing medications. While this treatment is generally effective, doctors have also introduced *quadruple therapy*, sequential therapies, and combination treatments to improve success rates

In this paper, we explore the most commonly used diagnostic methods—whether for an initial diagnosis or to confirm that the infection has been successfully eradicated—and discuss the key considerations for effective H. pylori treatment.Culturing is one of the most reliable methods for detecting *Helicobacter pylori* (H. pylori), with an accuracy that depends on several factors, including sample quality and handling. Under ideal conditions, culture sensitivity is typically above 90%, with a perfect specificity of 100%. However, sensitivity can vary—some studies report it as low as 85.4%, and in patients with active bleeding, it can drop to 40%. In individuals with stomach lining atrophy, culture accuracy remains high, with sensitivity at 96%, specificity at 100%, and an overall accuracy of 97%. Among children, sensitivity and specificity have been reported at 95.8% and 96.4%, respectively.



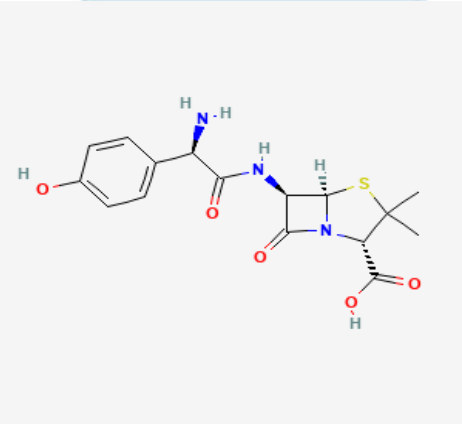


Fig 1: Chemical structure of vonoprazan

Fig 2 : chemical structure of amoxicillin

The understanding of *Helicobacter pylori* has evolved significantly, from its initial discovery to its recognition as a major public health concern. Ongoing research into its pathogenesis, diagnostic methods, and treatment options continues to inform clinical practice and public health strategies. Emphasis on personalized medicine and innovative therapies holds promise for more effective management and eradication of this pervasive infection.[22]

Hence the present study was undertaken to develop and validate a simple, precise, accurate, and stability-indicating RP-HPLC method for the simultaneous estimation of vonoprazan and amoxicillin in pharmaceutical dosage form. This method aims to provide releiable analytical tools for future perspective and quality assurance purposes.

II. Materials and Methods

1. Chemicals and reagents

Where vonoprazan and amoxicillin reference standards were purchased from yarrowchem pharmaceutical pvt ltd, and Leben life science pvt ltd. Akola. HPLC grade acetonitrile, water, and methanol were procured from merck (India) and reagent were of analytical or HPLC grade.

2. Instrumentation and Chromatographic Conditions:

- **Column:**Hypersil, C18 (250mm x 4.6ID, Particle size: 5 um)
- **Mobile phase:**MeOH:Water:Glacial acetic acid, 75:24.5:0.5
- **Flow rate :**1.0 ml/min
- **Detection wavelength:**244 nm
- **Injection volume:**20 µL.
- **Column temperature:**Ambient
- **Run time:**7 minutes

3. Detection of wavelength

The standard solution of vonoprazan and amoxicillin having strength 10 µg/ ml were prepared in methanol. These solutions were scanned individually and in combination using a UV visible spectrophotometer range (200 to 400 nm) to determine the wavelength of maximum absorbance. Both drugs showed satisfactory absorbance at 244 nm, which was selected at the detection wavelength for RP-HPLC analysis due to a good response and minimal baseline noise.

4. Preparation of standard stock solutions

Accurately weighed, 10 mg of vonoprazan and 250 mg of amoxicillin were transferred into 100 ml volumetric flask, dissolved in a diluent, and volume made up to the mark, to obtain the stock solution with a concentration of 10 and 250µg/ ml. From this stock solution, 2 ml was pipetted into a separate 20 ml volumetric flask and diluted with the same diluent to achieve a final concentration of 10 and 250 µg/ ml. The solution was shaken well and filtered through 0.2 µm nylon syringe filter before injecting into the HPLC system (10µg /ml and 250µg/ ml.)

5. Preparation of sample solutions

10 tablets were weighed individually and then crushed into a fine powder using a mortar and pestle. An accurately weighed quantity of powdered tablets, equivalent to required amount of active pharmaceutical ingredients, (APIs), was transferred to a 100 ml volumetric flask. The contents were dissolved in a diluent with vigorous shaking and sonicated for 2 minutes to ensure complete dissolution. From this solution, 2 ml was pipetted out into a 20 ml volumetric flask, diluted to volume with the same diluent, shaken, and sonicated. The final solution was filtered through a 0.2 µm membrane filter prior to HPLC analysis.

6. **Method validation**

The method was validated as per ICH Q2 (R1) guidelines for the following parameters:

6.1 system suitability

System suitability was assessed by injecting five replicates of standard solution before sample analysis. Parameters evaluated included : Retention time (RT), Theoretical Plates(TP), Tailing Factor(TF), Resolution(RS), % RSD of peak areas. All values were within acceptance limit, confirming suitability of the system.

6.2 Specificity

Specificity was confirmed by analyzing the drugs and sample solutions to check for interference at the retention times of the analytes.

6.3 **Accuracy**

Accuracy was assessed by recovery studies at 80%, 100%, and 120% levels. Known quantities of standard drugs were added to pre-analyzed samples, and percentage recovery was calculated.

6.4 Precision

Precision was evaluated in terms of :

Intraday Precision: Four replicates of sample solution were analyzed on the same day.

Interday Precision: The same procedure was followed on two different days. Results were expressed as %RSD, (Relative Standard Deviation.)

6.5 Linearity

Linearity was evaluated by preparing standard solution of vonoprazan and Amoxicillin. Calibration curves were plotted between peak area and concentration, and correlation coefficients R^2 were determined.

6.6 Limit of detection and limit of quantification.

LOD and LOQ were calculated using the formula:

- **LOD** = 3.3 (σ / S.)
- **LOQ** = 10 (σ /S,)

where Sigma is the standard deviation of the response and S is the slope of calibration curve.

6.7 Robustness

Robustness was evaluated by making small, deliberate changes in method parameters.

Flow rate (±0.1 ml per minute)

Mobile phase composition(±5%)

The effect on retention time, peak area, and resolution was observed.

6.8 Ruggedness.

Ruggedness was evaluated by analyzing the same sample under different conditions like change in batch or lot no of solvent . The %RSD of results was calculated to assess reproducibility under varied conditions.

III. Results and Discussions

1. Method Development

A reverse-phase HPLC method was successfully developed for the simultaneous estimation of Vonoprazan and amoxicillin in prepared tablets. After evaluating various solvent systems and detection wavelengths,mobile phase of methanol, water, and glacial acetic acid in the ratio of **75:24.5:0.5 v/v** and detection at 244 nm provided well-resolved, sharp, and symmetrical peaks for both analytes.

Retention time: vonoprazan - 2.5 amoxicillin - 3.4

The method showed good peak symmetry and baseline seperation.

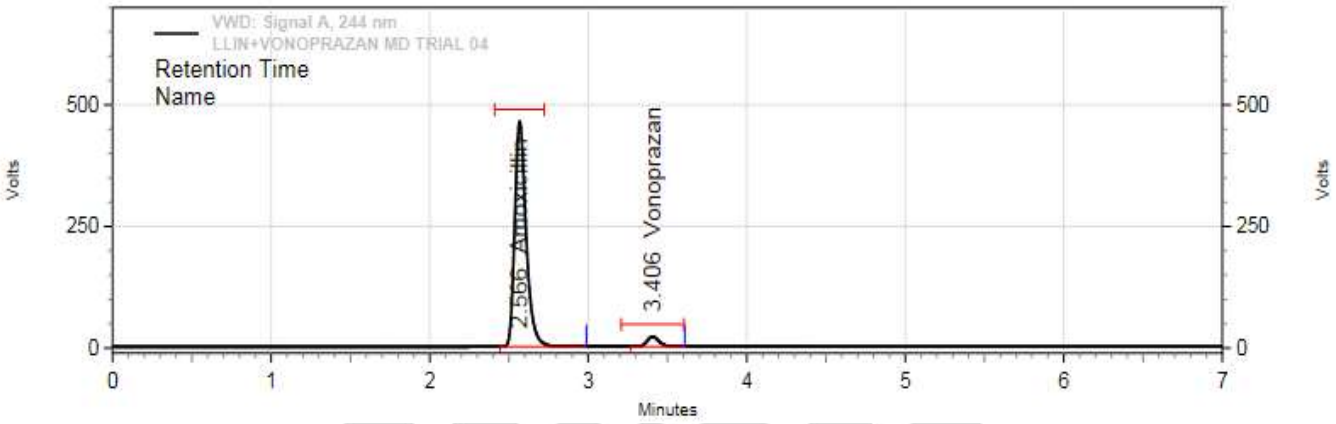


fig no 3 : Chromatogram showing resolved peaks of VONO & AMOX

2. System suitability

System suitability parameters were assessed before validation. The results are summerize below :

Table no.1: System suitability studies for vonoprazan

Name	Area	RT (min)	TP (NLT 2000)	TF (NMT 2)	Resolution (NLT 2)
Standard_Inj_01	1826233	3.401	9658	1.31	6.21
Standard_Inj_02	1822417	3.397	9764	1.38	6.23
Standard_Inj_03	1816933	3.401	9775	1.33	6.24
Standard_Inj_04	1814919	3.397	9791	1.37	6.21
Standard_Inj_05	1814919	3.397	9713	1.28	6.20
Mean	1819842	3.399			
SD	4512.8763	0.0022			
%RSD (NMT 2)	**0.25**	**0.06**			

Table no.2: System suitability studies for amoxicillin

Name	Area	RT (min)	TP (NLT 2000)	TF (NMT 2)	Resolution (NLT 2)
Standard_Inj_01	38751848	2.575	6535	1.46	6.21
Standard_Inj_02	38727298	2.571	6669	1.48	6.23
Standard_Inj_03	38727465	2.575	6550	1.45	6.24
Standard_Inj_04	38751922	2.579	6581	1.44	6.21
Standard_Inj_05	38738743	2.575	6561	1.45	6.20
Mean	38739455	2.575			
SD	12258.3872	0.0028			
%RSD (NMT 2)	**0.03**	**0.11**			

Remark: Theoretical plates, resolution and Tailing factor observed within acceptance criteria, also %RSD of replicate injections for area and retention time observed within acceptance criteria, hence system is suitable for analysis of both vonoprazan & Amoxicillin. Hence System Suitability is justified.

3. Specificity

There was no interferance from the blank or excipients. Well resolved and pure peaks confirmed the methods specificity.

4. Accuracy (Recovery Studies)

Accuracy was evaluated by spiking known quantities of drugs into the matrix. The recovery was within acceptable limits.

able no 3: Accuracy of vonoprazan:

Accuracy	Mean % recovery	SD	%RSD (NMT 2)
Accuracy at 80 %	99.34	0.9714	0.98
Accuracy at 100 %	100.00	1.3593	1.36
Accuracy at 120 %	99.82	1.2042	1.21

Table no 4: Accuracy of Amoxicillin

Accuracy	Mean % recovery	SD	%RSD (NMT 2)
Accuracy at 80 %	99.27	0.4851	**0.49**
Accuracy at 100 %	99.75	0.1826	**0.18**
Accuracy at 120 %	99.01	0.2152	**0.22**

5. Assay

% Assay of vonoprazan and amoxicillin in test solution 1 and 2 was found to be 99.50% and 99.02% and 99.59% and 99.84 % respectively.

Table no.5: % Assay of vonoprazan

Name	Area	RT(min)	% Assay
Test solutions-1	1810755	3.411	99.50
Test solutions-2	1838061	3.421	99.02

Table no 6: %Assay of Amoxicillin

Name	Area	RT(min)	% Assay
Test solutions-1	38767470	2.580	99.59
Test solutions-2	38910501	2.575	99.84

6. Precision

Precision was evaluated by intra-day repeatability studies. The %RSD values were below 2% for the both the drugs confirming the method's resproducibility under normal labouratory conditions.

able no 7: Intraday precision data of Vonoprazan

Name	Preparations	% Assay
Set-1	prep-1	99.50
	prep-2	99.02
Set-2	prep-1	98.26
	prep-2	99.24
Mean		99.01
SD		0.5340
% RSD (NMT 2)		**0.54**

able no 8 : Intraday precision data of Amoxicillin

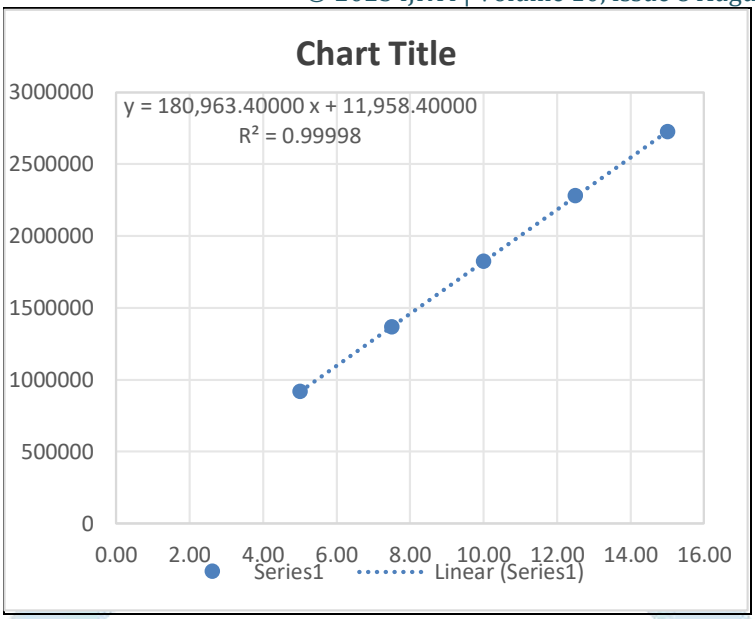
Name	Preparations	% Assay
Set-1	prep-1	99.59
	prep-2	99.84
Set-2	prep-1	99.68
	prep-2	99.86
Mean		99.74
SD		0.1297
% RSD (NMT 2)		**0.13**

7.Linearity

Linearity was evaluated by analyzing 5 different concentrations within accepted working range. A strong correlation between concentration and peak area was observed. Within correlation coefficients are square greater than 0.999 for both drugs. This confirms the method's reliability across the tested confirmation concentration range.

8.LOD & LOQ

LOD & LOQ were determined based on standard deviation and slope of the calibration curve. Theses values indicate the method's high sensitivity, capable of detecting and qualifying trace levels of both drugs.



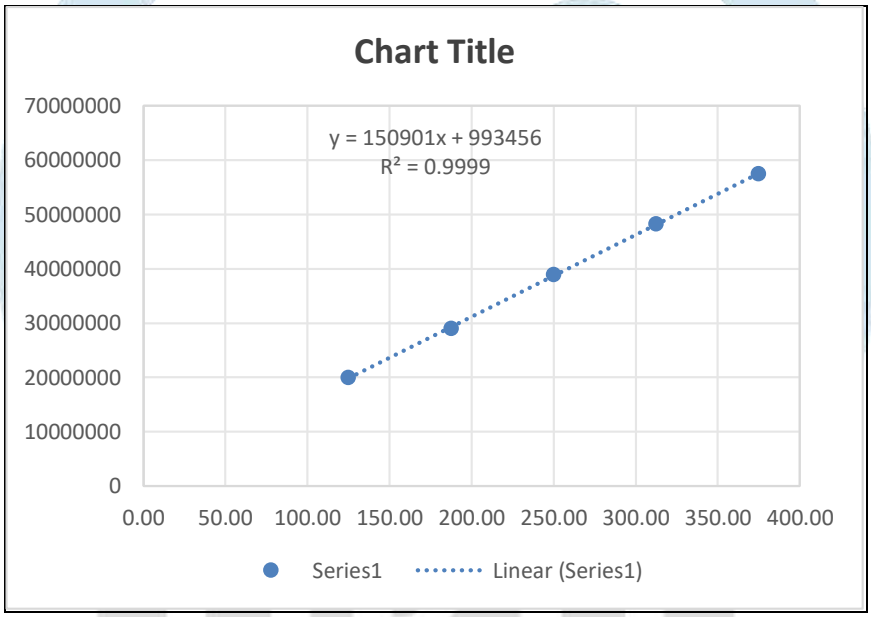


Fig no. 4 : linearity curve for vonoprazan

Fig no. 5 : Linearity curve of amoxicillin

Table no 9: LOD & LOQ of Vonoprazan

Con. (ppm or ug/ml)	Area
5.00	917839
7.50	1366071
10.00	1821418
12.00	2279434
15.00	2723200
STEYX	4110
SLOPE	180963.400
LOD (ug/ml)	**0.07**
LOQ (ug/ml)	**0.23**

Table no 10: LOD & LOQ of Amoxicillin

Con. (ppm or ug/ml)	Area
125.0	19958250
187.50	29023390
250.00	38857564
312.50	48255312
375.00	57498797
STEYX	197751
SLOPE	150900.82560
LOD (ug/ml)	**4.32**
LOQ (ug/ml)	**13.10**

9. Robustness

The robustness of the method was confirmed by deliberately altering flow rate and mobile phase. No significant variations were observed in retention time or peak area, proving that the method is reliable under slight changes in condition.

10. Ruggedness

Ruggedness was evaluated by changing batch/LOT number of solvent and performing the analysis on different days. The % RSD remained below 2%, indicating that the method is reproducible and rugged across varying operational environments.

Table no 11: Robustness changes in method parameters of Vonoprazan

Name	Preparations	%Assay
Original method parameters	Test prep-1	99.50
Original method parameters	Test prep-2	99.02
Pump, Flow 1.0ml/min	Test prep	99.71
Pump, Flow 1.2 ml/min	Test prep	99.94
Detector, 242nm	Test prep	99.16
Detector, 246nm	Test prep	98.92
Mean		99.38
SD		0.4065
%RSD (NMT 2)		**0.41**

Table no 12: Robustness changes in method parameters of Amoxicillin

Name	Preparations	%Assay
Original method parameters	Test prep-1	99.59
Original method parameters	Test prep-2	99.84
Pump, Flow 1.0 ml/min	Test prep	99.64
Pump, Flow 1.2 ml/min	Test prep	100.12
Detector, 242 nm	Test prep	99.45
Detector , 246 nm	Test prep	99.60
Mean		99.71
SD		0.2383
%RSD (NMT 2)		**0.24**

Table no 13: Ruggedness data for Vonoprazan

Name	Preparations	%Assay
Original method parameters	Test prep-1	99.50
Original method parameters	Test prep-2	99.02
Change in time	Test prep	99.87
change in day	Test prep	100.76
Change in Batch or Lot of solvent	Test prep	98.87
Mean		99.60
SD		0.7579
%RSD (NMT 2)		**0.76**

able no 14: Ruggedness data for Amoxicillin

Name	Preparations	%Assay
Original method parameters	Test prep-1	99.59
Original method parameters	Test prep-2	99.84
Change in time	Test prep	100.03
change in day	Test prep	101.03
Change in Batch or Lot of solvent	Test prep	99.75
Mean		100.06
SD		0.5717
%RSD (NMT 2)		**0.57**

CONCLUSION : A simple, precise, accurate, and robust reverse-phase HPLC method was successfully developed and validated for the simultaneous estimation of vonoprazan and amoxicillin in pharmaceutical dosage form. The method demonstrated excellent linearity, precision, accuracy, specificity, and system suitability as per ICH guidelines. Low values of LOD and LOQ indicate the method's high sensitivity. The developed method was effectively applied to the analysis of tablets and the assay results were within acceptable limits. Overall, the method is reliable and suitable for routine quality control analysis of vonoprazan and amoxicillin in pharmaceutical dosage form.

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