IJMPR 2022, 6(3), 70-76

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article
SJIF Impact Factor: 5.273

# DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CLARITHROMYCIN, AMOXICILLIN AND ESOMEPRAZOLE IN FIXED DOSE COMBINATIONS

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**Received on: 26/01/2022**
**Revised on: 16/02/2022**
**Accepted on: 08//03/2022**

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## ABSTRACT

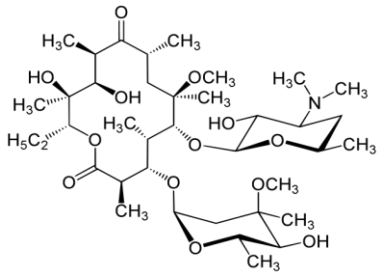
New RP-HPLC method was developed for the simultaneous estimation of Clarithromycin, amoxicillin and esomeprazole in the fixed dose combination (PYLOKIT AC by CIPLA). RP-HPLC separation was carried out using Ultisil XB-CN column (250 x 4.6 mm) internal diameter and the packing material having 5µm size) using gradient mobile phase of Potassium dihydrogen phosphate: acetonitrile. 1 mL/min was the flow rate and UV detector at 210 nm wavelength was fixed for detection of the drug. The method validation was done as per ICH guideline and the parameters were included such as accuracy, precision specificity, linearity, and robustness were determined. Retention times for the clarithromycin, amoxicillin and esomeprazole were 16.481, 3.747 and 10.586 minutes respectively. RP-HPLC method was a simple, reliable and acceptable and it confirmed that method is suitable for the intended use for routine quality control and assay of drugs. This method is successfully applied for the determination of commercial dosage form preparation. This method is validated as per ICH (International council on harmonization) Guidelines.

**KEYWORDS:** RP-HPLC, Clarithromycin, Amoxicillin, Esomeprazole, Method Development, Method Validation.

## INTRODUCTION

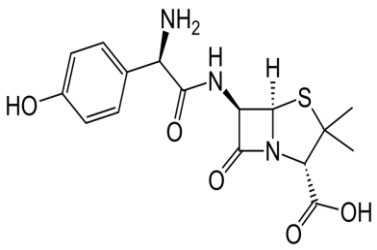
Peptic ulcers are localized erosions of the mucous membranes of the stomach and duodenum. The pain associated with ulcers is caused by irritation of exposed surfaces by the stomach acids. The current approach for treating ulcers caused by Helicobacter pylori is to use combination of drugs, which includes a proton pump inhibitor and two antimicrobials, such as tinidazole and amoxicillin or clarithromycin.

### Structure of Clarithromycin



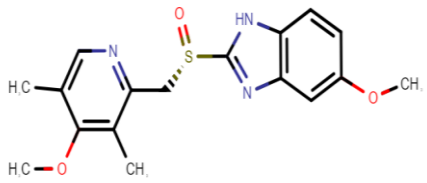
Clarithromycin is a semi-synthetic macrolide antibiotic derived from erythromycin A. It consists of a 14 membered lactone ring as well as cladinose and desosamine residues at positions 3 and 5 of the ring, respectively. Like erythromycin, it has no conjugated double bond in the lactone ring, hence significant UV absorbance is only obtained at wavelengths below 210 nm.[1-2] It is white or almost white crystalline powder, practically insoluble in water, slightly soluble in alcohol and acetonitrile and freely soluble in acetone. It may be bacteriostatic or bactericidal depending on the organism and drug concentration and it mainly acts by inhibiting bacterial protein synthesis. The drug is used to treat pharyngitis, tonsillitis, acute maxillary sinusitis, acute bacterial exacerbation of chronic bronchitis, pneumonia, skin infections, etc.[3] Literature survey revealed that various methods have been developed for the estimation of clarithromycin from laboratory prepared mixtures, pharmaceutical preparations and biological matrices (such as human plasma) through automated solid phase extraction and electrochemical detection, liquid chromatographic electrospray tandem mass spectrometry and high performance liquid chromatography.[4-7]

### Structure of Amoxicillin



Amoxicillin trihydrate chemically recognized as [2S-[2a,5a,6aˆ(S)]]-6-[[Amino(4-hydroxyphenyl)acetyl]amino]-3,3 dimethyl-7-oxo-4-thia-1-azabicyclo is the most extensively used β-lactam antibiotic to treat bacterial infections of ear, nose, throat, skin and lower respiratory tract due to susceptible microorganisms. It has significant absorption ability than other β-lactam antibiotics. The marketed formulations of amoxicillin are capsules, suspensions, tablets and injectable solutions. The combination drugs of Amoxicillin enhances the antibacterial effect and bacterial resistance. In literature, numerous analytical techniques have been employed for quantitative determination of amoxicillin by HPLC.[7-10]

**Structure of Esomeprazole**



Esomeprazole belongs to class of proton pump inhibitors (PPI). It is a S isomer of omeprazole that act by hindering enzymatic action in parietal cells of gastric mucosa, hence, reducing hydrogen ion movement into gastric lumen. Esomeprazole is used in different clinical situations such as gastro oesophageal reflux disease (GERD), stomach and intestinal ulcers and heartburn.[11-12]

Various HPLC methods have been reported for analysis of amoxicillin, clarithromycin and esomeprazole. However, no isocratic RP-HPLC method has been reported for simultaneous determination of amoxicillin, clarithromycin and esomeprazole. Therefore, this study aims at developing and validating a simple, precise, accurate and robust RP-HPLC method for simultaneous determination of amoxicillin, clarithromycin and esomeprazole.

## MATERIALS AND METHODS

Clarithromycin, amoxicillin and esomeprazole were the gifted samples obtained from Uttranchal Research and Testing laboratory, Uttrakhand. The Samples were characterized by Infrared Spectroscopy (IR) and Mass Spectroscopy for checking the purity level.

RANKEM Chemicals supplied the AR grade chemicals of Potassium dihydrogen phosphate, orthophosphoric acid (OPA), methanol and HPLC grade acetonitrile. Milli-Q water purification system produced water was used during analysis (Make & Model: MILLIPORE / Integral 5).

**Instrumentation and Software**

The validated analytical method was performed on HPLC (Make & Model: Agilent with UV Detector) which is equipped with solvent delivery pump, degasser, using Openlab®Software.

RP-HPLC separation was carried out using Ultisil XB-CN column (250 x 4.6 mm internal diameter and the packing material having 5µm size) using gradient programming mobile phase of buffer: acetonitrile (The pH was adjusted to 4.0 by the help of orthophosphoric acid). 1 mL/min was the flow rate and UV detector at 210 nm wavelength was fixed for detection of the drugs.

**Methods**

| **Chromatographic conditions** | | | |
|---|---|---|---|
| **Column** | **Ultisil XB-CN, column (250mmx4.6mm), 5 µm** | | |
| **Mobile phase** | **Gradient programming** | | |
| | **Time** | **Buffer ($KH_2PO_4$)** | **Acetonitrile** |
| | 0 | 90 | 10 |
| | 17 | 70 | 30 |
| | 20 | 70 | 30 |
| | 21 | 90 | 10 |
| | 25 | 90 | 10 |
| **Detector** | UV detector | | |
| **Flow rate** | 1 ml/min | | |
| **Wavelength** | 210nm | | |
| **Injection Volume** | 5µL | | |
| **Temperature** | 30°C | | |
| **Diluent** | Methanol | | |

**Preparation of Standard Solution**

The stock solutions were prepared by dissolving 50mg of esomeprazole in 50 ml flask, add approx. 10 ml of diluent and mix well. Make the volume.

Take 1 ml of solution from this solution in 100ml flask. Add 12.5mg of clarithromycin and 20 mg of amoxicillin. Mix well with diluent and finally make the volume to prepare final concentrations of 125µg/mL for Clarithromycin, 200 µg/mL for Amoxicillin and 10

µg/mL for Esomeprazole respectively. The solution was filtered through 0.45µm membrane filter.

**Preparation of Sample Solution**

The sample solutions were prepared by dissolving 1/10 of average weight of drugs in 100ml flask and mix well with diluents to prepare final concentrations of 125µg/mL for Clarithromycin, 200 µg/mL for Amoxicillin and 10µg/mL for Esomeprazole respectively. The solution was filtered through 0.45µm membrane filter.

**Method Validation**

The developed method was validated with respect to system suitability, specificity, linearity, precision, accuracy LOD, LOQ and robustness in the accordance of the ICH Q2 guidelines.

**Specificity and selectivity**

The developed method was found to be selective for Clarithromycin, amoxicillin and esomeprazole, since the injection of the blank solution confirmed the absence of interfering peak at RT examined substance at 210nm.The results obtained demonstrate that there was no interference from other material in the developed method and therefore confirm the specificity of the method.

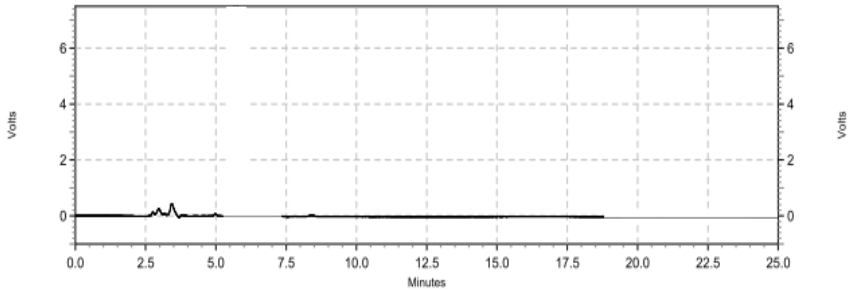
**System Suitability**

System Suitability tests are an integral part of method development and were used to ensure adequate performance of the chromatographic system. Retention Time (RT), tailing factor, peak asymmetry, and theoretical plates (T) were evaluated. The results are shown here in Table 1.

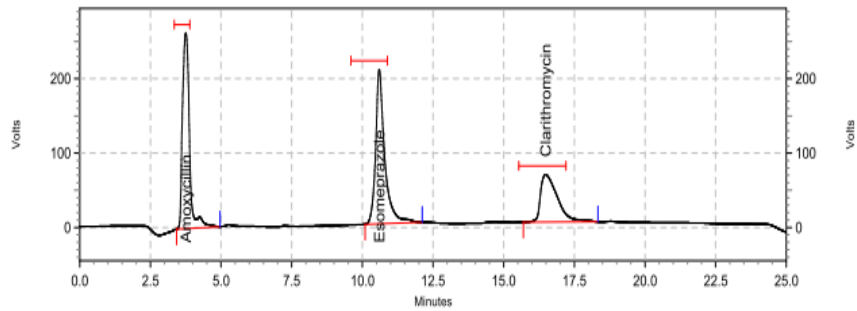
**Table 1: System suitability parameters of Clarithromycin, amoxicillin and esomeprazole.**

| Sr. No. | Property | Clarithromycin | Amoxicillin | Esomeprazole | Acceptance criteria |
|---|---|---|---|---|---|
| 1. | Retention Time (RT) | Retention Time (RT) | 16.481 | 3.747 | 10.586 |
| 2. | Tailing factor (T) | Tailing factor (T) | 1.31 | 1.24 | 1.12 |
| 3. | Theoretical plates (N) | Theoretical plates (N) | 3470 | 4254 | 2830 |

From the data it was found that all the system suitability parameters for developed method were within the limit.




**Chromatogram: Blank**




**Chromatogram: Standard**

**Linearity and Range**

Linearity of the developed method demonstrates the ability of method to produce a result which is directly proportional to concentration of analyte in the sample. The amount of clarithromycin, tinidazole and lansoprazole were prepared for linearity in the range of 80-120%.The amount of clarithromycin, amoxicillin and esomeprazole in five different concentrations is 80%, 90%, 100%, 110% and 120% of working strength respectively. The graph was plotted between concentrations versus area of peak. The clarithromycin, amoxicillin and esomeprazole shows good correlation coefficients ($R^2$= 0.9994, 0.9986 and 0.9993) and the proposed method was linear in concentration range 80-120 %.

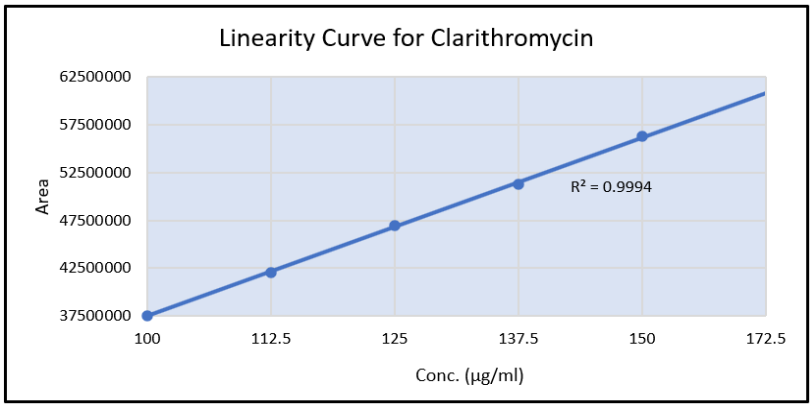
**Table 2**: **Linearity of Clarithromycin, Amoxicillin and Esomeprazole.**

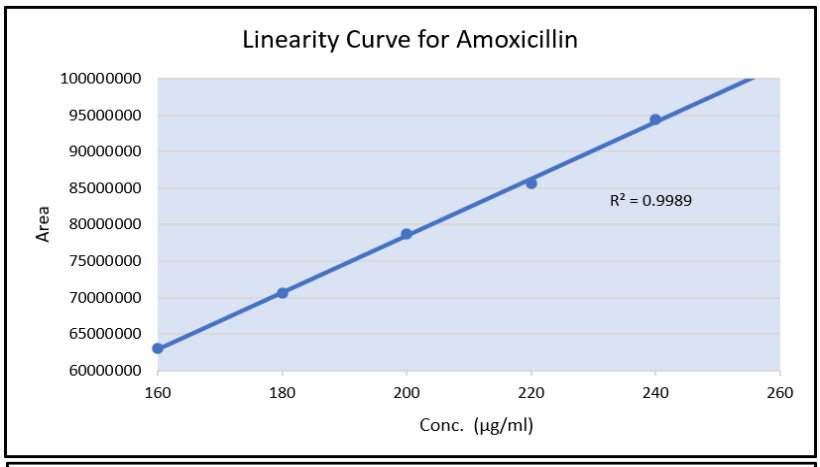
| S. No. | Compound | Values of X and Y Variables | | | | | | Correlation co-efficient |
|---|---|---|---|---|---|---|---|---|
| 1 | Clarithromycin | **Variable** | **1** | **2** | **3** | **4** | **5** | 0.9994 |
| | | X | 100 | 112.5 | 125 | 137.5 | 150 | |
| | | Y | 37555118 | 42036419 | 46943897 | 51240328 | 56332676 | |
| 2 | Amoxicillin | X | 160 | 180 | 200 | 220 | 240 | 0.9996 |
| | | Y | 62963590 | 70634048 | 78704488 | 85594456 | 94445386 | |
| 3 | Esomeprazole | X | 8 | 9 | 10 | 11 | 12 | 0.9993 |
| | | Y | 65081218 | 73016371 | 81351523 | 88686676 | 97621828 | |

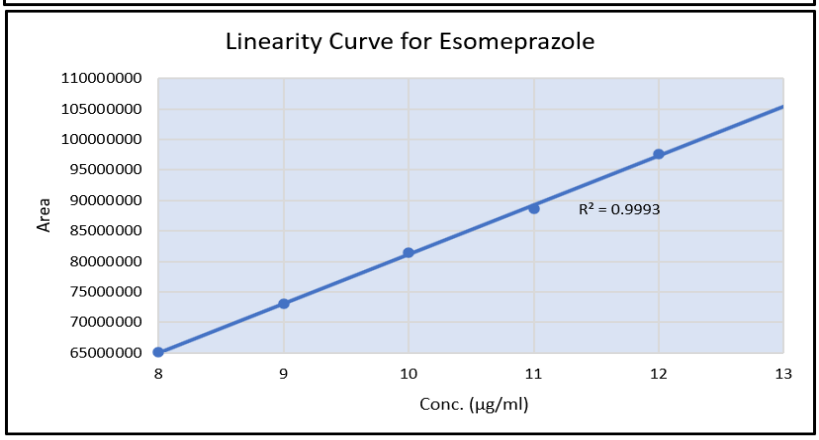
Note: X is the concentration of the respective component in µg/mL. Y is the peak response of the respective component in area counts.

**Linearity Curve**

Calibration curve was constructed between concentrations versus peak area. Results were recorded for equation of line and correlation co-efficient were determined.



**Precision**

It reveals the data regarding closeness between the series of measurements. The precision of the developed method was verified by system precision and method precision. A homogenous sample concentration of 125 µg/mL for Clarithromycin, 200 µg/mL for Amoxicillin and 10 µg/mL for Esomeprazole respectively were prepared under prescribed conditions and estimation was carried out. The results are expressed in the form of standard deviation and RSD value. Table 3 and 4 shows the result of system precision and method precision respectively and the developed method is highly precise as % RSD is less than 2%.

**Table 3**: **Calculation of %RSD for Clarithromycin, amoxicillin and Esomeprazole (System Precision).**

| S. No. | Compound | No. of Injections | | | | | | Mean | S.D. | %RSD |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | | | |
| 1 | Reference Standard Clarithromycin | 46933897 | 46923482 | 46912560 | 46932869 | 46922551 | 46933482 | 46926473.5 | 8521.15 | 0.018 |
| 2 | Reference Standard Amoxicillin | 78704488 | 78672961 | 78683934 | 78693957 | 78692992 | 78683168 | 78688583.3 | 10920.35 | 0.014 |
| 3 | Reference Standard Esomeprazole | 81301523 | 81279133 | 81288152 | 81299120 | 81289087 | 81299285 | 81292716.7 | 8720.36 | 0.010 |

**Table 4**: **Calculation of %RSD for Clarithromycin, Amoxicillin and Esomeprazole (Method Precision).**

| S. No. | Compound | No. of Injections | | | | | | Mean | S.D. | %RSD |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | | | |
| 1 | Sample Clarithromycin | 46754815 | 46775051 | 46785513 | 46795520 | 46805139 | 46743942 | 46776663.3 | 23644.82 | 0.050 |
| 2 | Sample Amoxicillin | 78502635 | 78482590 | 78512956 | 78523067 | 78542981 | 78502632 | 78511143.5 | 20587.49 | 0.026 |
| 3 | Sample Esomeprazole | 81202477 | 81222365 | 81242831 | 81232089 | 81253071 | 81305217 | 81243008.3 | 35098.07 | 0.043 |

**Mean** represents the average values of six replicates analysis. **SD** is the standard deviation calculated on the six replicates. **RSD** is the relative standard deviation.

**Table 5: System Precision and Method precision.**

| Precision | Drug | % RSD |
|---|---|---|
| System precision | Clarithromycin | 0.018 |
| Method precision | Clarithromycin | 0.050 |
| System precision | Amoxicillin | 0.014 |
| Method precision | Amoxicillin | 0.026 |
| System precision | Esomeprazole | 0.010 |
| Method precision | Esomeprazole | 0.043 |

**Accuracy**

It is also termed as trueness or recovery. This method was determined using 80%, 100% and 120% of working strength of Clarithromycin, amoxicillin and esomeprazole. Each level solution was prepared in duplicate and analysed as per the method given. This is usually demonstrated in the form of SD and RSD. The results reveal that the value of % RSD is less than 2%. The percent recovery results are in Table 6.

**Table 6: Summary of assay of Clarithromycin, amoxicillin and esomeprazole.**

| S. No. | Level | Compound | % Average Assay | %RSD |
|---|---|---|---|---|
| 1 | 80% | Clarithromycin | 98.41 | 0.24 |
| | | Amoxicillin | 99.54 | 0.19 |
| | | Esomeprazole | 99.61 | 0.17 |
| 2 | 100% | Clarithromycin | 98.93 | 0.20 |
| | | Amoxicillin | 99.29 | 0.21 |
| | | Esomeprazole | 99.71 | 0.12 |
| 3 | 120% | Clarithromycin | 98.92 | 0.23 |
| | | Amoxicillin | 99.25 | 0.17 |
| | | Esomeprazole | 99.81 | 0.13 |

The percentage of assay values of clarithromycin was in the range of 99.41-99.93 %, amoxicillin in the range of 99.24-99.54 % and esomeprazole in the range of 99.61-99.81%. The % RSD of assay values of clarithromycin were in the range of 0.20-0.24 %, amoxicillin in the range of 0.17-0.21 % and Esomeprazole in the range of

0.12-0.17%. The study proves that the method is accurate for the estimation of amoxicillin, clarithromycin and lansoprazole assay over the range of 80-120% of target concentration.

**LOD and LOQ (Limit of Detection and Limit of Quantification)**

Limit of detection (LOD) and Limit of Quantification (LOQ) reveal information regarding concentration of analyte that yields signal-to-noise around 1 to 10. Serial dilutions are made from solution of clarithromycin, amoxicillin and esomeprazole for determination of LOQ and LOD. The samples were injected in HPLC and compare the signals of sample and blank sample of LOD and LOQ. According to earlier mentioned parameters, LOD and LOQ were estimated for clarithromycin, amoxicillin and esomeprazole were 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, and 2 µg/ml and 4 µg/ml respectively.

**Robustness**

The robustness of the developed HPLC method was carried out by making small deliberate changes in the HPLC process parameters. These parameters include variation in wavelength, flow rate of mobile phase and changes in proportion of buffer and acetonitrile. The method was performed on single concentrations of clarithromycin, amoxicillin and esomeprazole. The alteration of parameters may leads to some significant changes in the peak area and RSD. Robustness studies concludes that the method is robust under ± 2 wavelength, ± 10% flow rate and ± 10% increase and decrease in mobile phase and at the different column (Zorbax CN column (250mmx4.6mm), 5 micron. There is no significant change in recovery of amoxicillin, tinidazole and omeprazole. The % RSD shown in table:7 negligible changes were observed during robust condition. So, we can say that the developed method is robust.

**Table 7: Robustness Data.**

| Drug | Parameters | % RSD |
|---|---|---|
| **Clarithromycin, amoxicillin and esomeprazole** | Wavelength minus | 0.005 |
| | Wavelength plus | 0.003 |
| | Flow minus | 0.002 |
| | Flow plus | 0.003 |
| | Mobile phase ratio change | 0.002 |
| | Column Change | 0.001 |
| | Temperature minus | 0.004 |
| | Temperature plus | 0.003 |

## RESULT AND DISCUSSION

After a number of trials with different, mobile phases were tested but the adequate separation of Clarithromycin, amoxicillin and esomeprazole was found in Potassium dihydrogen phosphate: Acetonitrile (Gradient programming). The best results were obtained with flow rate gradient programming of selected mobile phase for the purpose of rapid analysis. Mobile phase was started at a flow rate of 1.0 ml/min which was continued for 1.0 min to 25.00 min.

The validation of the developed and the optimized RP-HPLC method was carried out with respect to the parameters such as specificity, linearity, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD) in the light of internationally accepted ICH guidelines.

## CONCLUSION

The HPLC method was successfully developed and validated on an Agilent 1220 LC for simultaneous determination of clarithromycin, amoxicillin and esomeprazole in PYLOKIT AC combination. This present method is simple and accurate for the determination of drug at a single wavelength, 5µL injection capacity and Ultisil XB C18 5µm 4.6*150mm column. It was found that the method is sufficiently simple, rapid and sensitive as well as precise, accurate, linear, robust which compiles the ICH guidelines. The entire experimentation was proved that the developed HPLC method shows good resolution, linearity and RSD values (less than 2%) which indicate that method is suitable for the estimation of Clarithromycin, amoxicillin and esomeprazole.

## ACKNOWLEDGEMENT

Authors are thankful to Uttrakhand Research and Testing Laboratory, Uttrakhand for the necessary instruments and facility provided.

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