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Research

# Simultaneous Determination of Amoxicillin, Clarithromycin and Esomeprazole in Mice Plasma after Oral Administration by Reverse Phase HPLC Method

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## ABSTRACT

A simple and precise reverse phase high-performance liquid chromatography (RP-HPLC) method for simultaneous determination of amoxicillin, clarithromycin and esomeprazole in mice plasma after an oral administration was developed and validated. An isocratic elution was achieved on C18 column with a mobile phase containing buffer, potassium di hydrogen phosphate ($KH_2PO_4$) 0.05 M at pH 5 and methanol (60:40 v/v) at a flow rate of 1 mL/min, injection volume was 10 µL and UV detection was kept at 205 nm. Linearity was in the range of 0.5-100 µg/mL for amoxicillin, 50-1000 µg/mL for clarithromycin and 0.1-100 µg/mL for esomeprazole. Limit of detection (LOD) and Limit of quantification (LOQ) were 0.26 µg/mL and 0.79 µg/mL for amoxicillin, 8.97 µg/mL and 27.20 µg/mL for clarithromycin, 0.13 µg/mL and 0.39 µg/mL for esomeprazole respectively. All samples were stable at room temperature for 72 hours. The pharmacokinetic studies showed that the maximum plasma concentrations ($C_{max}$) were 1042.17 ± 4.0, 218.67 ± 5.3 and 18.97 ± 3.6 µg/mL for amoxicillin, clarithromycin and esomeprazole respectively. Whereas, the times to reach maximum plasma concentration ($T_{max}$) were 2.0, 4.0 and 2.0 hours respectively. Over all, the validated HPLC method may be used for the determination of such drugs in their pharmaceutical formulation and can be applied for routine quality control analysis.

**Keywords:** HPLC; Amoxicillin; Clarithromycin; Esomeprazole; Pharmacokinetics

## INTRODUCTION

Helicobacter pylori (*H. Pylori*) infection is one of the most common infections globally. Approximately, 50% of the world population is known to be infected by *H. Pylori*. Among these millions of patients develop peptic ulcer, and many of these cases progress to gastric cancer [1]. Helicobacter pylori (*H. pylori*) is a gram negative bacteria, considered as one of the main causative agents of peptic ulcer [2]. Antibiotic regimens can eradicate the bacterial infection successfully and reduce the chances of recurrence. Triple therapy comprising of two antibiotics amoxicillin, clarithromycin and a proton pump inhibitor (PPI) are considered as a standard therapy for treatment of *H. pylori* [3].

Amoxicillin tri hydrate belongs to penicillin class of antibiotics that is degraded by β lactamase enzyme, produced by bacteria [4]. The peak plasma concentration of amoxicillin has been achieved in 60-90 minutes after administration and bioavailability ranges from 70-90%. Amoxicillin is metabolized in the liver mainly by hydrolysis and excreted unchanged in urine after 6 hours of administration [5]. The half-life of amoxicillin is approximately 1 hour and its protein binding is less than 25% which means that such drugs are well distributed in the body [6]. Amoxicillin is used in the treatment of pneumonia and also for the eradication of *H. pylori* in combination with other antibiotics [7].

Clarithromycin is a broad spectrum antibiotic, a second generation macrolide active against bacterial infections [8]. It inhibits the bacterial protein synthesis by binding to 50 S ribosomal subunit. Clarithromycin remains stable in gastric environment; as a result it has better bioavailability [9]. Clarithromycin is mainly used for respiratory tract infection; however, it is also included as a component of *H. pylori* regimens [10].

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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 [11]. Esomeprazole is used in different clinical situations such as gastro oesophageal reflux disease (GERD), stomach and intestinal ulcers and heartburn.

High performance liquid chromatography (HPLC) is the most commonly used analytical technique for quantification of drugs. Various HPLC methods have been reported for analysis of amoxicillin [12], clarithromycin [13,14] and esomeprazole [15,16]. 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. The pharmacokinetic study of these triple combinations was further evaluated in mice plasma after oral administration.

## MATERIALS AND METHODS

### Reagents and materials

The standards of Amoxicillin tri hydrate, clarithromycin and esomeprazole magnesium tri hydrate were purchased from Sigma Aldrich, Germany. Buffering agents Potassium dihydrogen phosphate ($KH_2PO_4$), were also obtained from sigma, Aldrich. HPLC grade methanol and acetonitrile were purchased from DUKSAN pure chemicals, South Korea. Sodium hydroxide (NaOH) was obtained from Merck, Germany. Distilled water was obtained from laboratory distillation apparatus. All the reagents used were of analytical grade.

### Instrumentation

Liquid chromatographic system consisted of a Quaternary pump (Agilent technologies 1260 infinity, 1200 infinity series) with a data system (Chemstation for LC system), diode array detection (DAD) with multiple wavelength detection, auto sampler equipped with vacuum degasser. Column (stationary phase) used was a SUPELCOSIL LC-1 reverse phase C18 (25 cm, 4.6 mm, 5 µm). Mobile phase was a mixture of methanol and buffer at pH 5 (40:60 v/v). Mobile phase was filtered through 0.22 µm nylon filter and degassed by sonication in bath sonicator for 15 minutes. Detection was carried out at 205 nm. The flow rate was 1 mL/min and injection volume was 10 uL.

### Preparation of mobile phase

Buffer solution of 0.05 M was prepared by dissolving 6.8 g of potassium di hydrogen phosphate in 1000 mL distilled water. Dilute ortho phosphoric acid was used to adjust the pH at 5. Mobile phase was a mixture of buffer and methanol (60:40 v/v), filtered through 0.22 µm nylon filter and degassed by sonication prior to use.

### Preparation of standard solutions

Stock solutions of Amoxicillin, Clarithromycin and Esomeprazole were prepared by accurately weighing on electronic balance and then dissolved in methanol by stirring having concentration of 1 mg/mL. Aliquots of standard stock solutions were taken into vials and volume was made up to mark with methanol to prepare the desired concentrations ranging from 0.01-100 µg/mL for amoxicillin and esomeprazole and 50-1000 µg/mL for clarithromycin. All solutions were filtered through 0.22 µm nylon filter and degassed by sonication before analysis. Figure 1 shows original chemical structure of Amoxicillin, Clarithromycin and Esomeprazole.

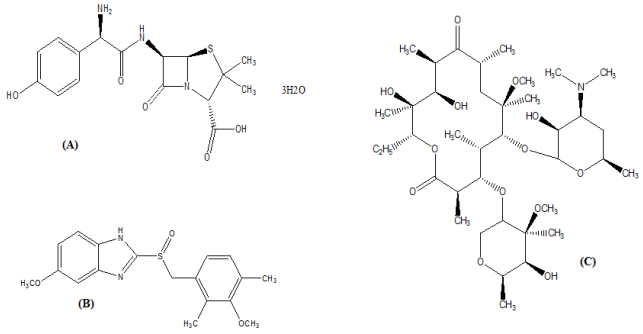


**Figure 1**: Structures of (A) Amoxicillin, (B) Esomeprazole and (C) Clarithromycin

### Validation parameters

**Linearity**: Method of linearity was evaluated by plotting Calibration curves of amoxicillin in concentration range (0.5-100 µg/mL), esomeprazole (0.1-100 µg/mL) and for clarithromycin in concentration range (0-1000 µg/mL). Calibration curves were constructed by plotting peak area versus concentration as presented in Table 1. Regression equations were y=25.975x+109.87 ($R^2$=0.9994) for amoxicillin, y=0.7001x +3.0218 ($R^2$=0.9996) for clarithromycin and y=52.039x +49.527($R^2$=0.9996) for esomeprazole. RSDs % for linearity data were <2%.

**Limit of detection (LOD) and limit of quantification (LOQ)**: Different terms have been used for LOD and LOQ but generally LOD is stated as a lowest concentration of analyte that can be detected but not quantified. LOQ is defined as the lowest concentration of analyte in a sample that can be detected and quantified with adequate accuracy and precision [17]. Results of LOD and LOQ for amoxicillin, clarithromycin and esomeprazole were presented in Table 2. Limit of detection for amoxicillin, clarithromycin and for esomeprazole were 0.26169 µg/mL, 8.976 µg/mL and 0.13157 µg/mL respectively. Limit of quantification was 0.79302 µg/mL, 27.200 µg/mL and 0.39872 µg/mL for amoxicillin, clarithromycin and esomeprazole respectively.

Value for LOD and LOQ can be calculated by following formulas

$LOD=3.3\ \sigma/S$ (1)

$LOQ=10\ \sigma/S$ (2)

Where,

σ=Standard deviation of response

S=Slope of calibration curve

**Accuracy and precision**: Accuracy and precision of amoxicillin and esomeprazole was determined at three different concentrations of 5 µg/mL, 50 µg/mL and 100 µg/mL in triplicate injections. For clarithromycin both accuracy and precision was determined at 50 µg/mL, 100 µg/mL and 1000 µg/mL. The inter day and intraday precision for all three compounds as shown in Table 3, relative standard deviation (RSD%) were <2%. Accuracy was in the range of 98-102% for amoxicillin, clarithromycin and esomeprazole. These values of accuracy and precision were within limits, showing that the developed method was accurate and precise for determination of amoxicillin, clarithromycin and esomeprazole.

**Recovery studies**: Recovery studies were determined by standard addition method. Standard working solutions containing amoxicillin, clarithromycin and esomeprazole were prepared at three different concentrations (at level of 50,100 and 150%). Final concentrations were 30, 40 and 50 µg/mL for amoxicillin and esomeprazole. Recovery study of clarithromycin was determined at concentrations 750, 1000 and 1250 µg/mL. The prepared mixtures were injected in triplicate. Percentage recovery and RSD% were calculated for amoxicillin, clarithromycin and esomeprazole as given in Table 4, results are close to 100% and RSD% was <2%.

**Robustness**: Robustness of method was assessed by slight changes in experimental conditions. Solutions of three drugs were tested at flow rate of (0.96, 0.98 and 1 mL/min). Standard solution of amoxicillin, clarithromycin and esomeprazole were also tested by changing the organic compositions of mobile phase, buffer and methanol in ratios of 62:38, 60:40 and 58:42 v/v and changing the pH (4.8, 5.0 and 5.2) of mobile phase. Data obtained from robustness as presented in Table 5 showed that slight changes in pH of mobile phase, flow rate and composition of mobile phase have no significant difference in peak area, retention time, symmetry and resolution of peaks.

### Pharmacokinetic study

**Animals**: Adult male BALB/c mice weighing (30 ± 5) g were purchased from the national institute of health (NIH), Pakistan. Animals were kept at conditions of room temperature (25 ± 1) $^{o}$C and maintained with free access to water. All the experiments was carried out according to the NIH guidelines for care and use of laboratory animals and approved by animal ethics committee of Quaid-i-Azam University.

**Dosing and sampling**: Mice were fasted overnight before dosing. Oral dose of amoxicillin, clarithromycin and esomeprazole according to body weight was calculated and required amount of three drugs was dissolved in small volume of dimethyl sulfoxide (DMSO) while final volume was adjusted with normal saline. Oral dose of amoxicillin (50 mg/kg), clarithromycin (15 mg/kg) and esomeprazole (0.7 mg/kg) was delivered as a single dose (300 µL) by using a ball tipped oral gavage needle. About 0.3 mL blood samples were collected in eppendorf tubes at approximately after 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours and 24 hours. Blood samples were centrifuged at 13000 rpm for 10 minutes. Plasma was obtained and stored in refrigerator for further HPLC analysis.

**Data analysis**: The pharmacokinetic parameters such as area under the plasma concentration-time curve (AUC), maximum plasma drug concentration ($C_{max}$), time to reach $C_{max}$ ($T_{max}$), half-life (t1/2), elimination rate constant ($K_e$), were calculated using the trapezoidal rule-extrapolation method [18] and also Microsoft Excel was used. All the results are expressed as mean ± standard deviation of triplicates.

## RESULTS AND DISCUSSION

### Optimization of chromatographic conditions

The choice of mobile phase was based on the type of chromatography. Since, reverse phase chromatography is more efficient for pharmaceutical analysis; therefore, polar mobile phase was used. Different solvents such as acetonitrile, methanol and buffer in different ratios were tested to select optimum composition of mobile phase. The optimum composition of mobile phase was buffer and methanol in 60:40 v/v and pH 5 at flow rate of 1 mL/min, showed good resolution of the three drugs. The retention times for amoxicillin, clarithromycin and esomeprazole were about 3.1, 4.2 and 7.2 minutes respectively as shown in Figure 2. Solutions of the three drugs were scanned at 205, 210, 220 and 230 nm. The wavelength selected as shown in Figure 3, was 205 nm at which these three drugs showed maximum absorbance. Furthermore, the obtained data showed close resemblance to the already reported results [19].

| **Amoxicillin concentrations (µg/mL)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| Concentration | 0.5 | 1 | 5 | 10 | 20 | 50 | 100 |
| Mean area | 112.166 | 130.433 | 231.366 | 375.633 | 622.7 | 1456.366 | 2684.766 |
| SD ± | 1.65 | 1.55 | 1.15 | 3 | 2 | 3.62 | 1.41 |
| RSD% | 1.47 | 1.19 | 0.5 | 0.79 | 0.32 | 0.24 | 0.05 |
| $R^2$ | 0.9994 | | | | | | |
| **Clarithromycin concentrations (µg/mL)** | | | | | | | |
| Concentration | | 50 | 100 | 125 | 250 | 500 | 1000 |
| Mean area | | 37.166 | 73.8 | 94.8 | 178.033 | 351.966 | 705.566 |
| SD ± | | 1.33 | 1.25 | 1.47 | 1.44 | 6.13 | 1.8 |
| RSD% | | 0.03 | 1.69 | 1.55 | 0.81 | 1.74 | 0.25 |
| $R^2$ | 0.9996 | | | | | | |
| **Esomeprazole concentrations (µg/mL)** | | | | | | | |

| Concentration | 0.1 | 1 | 5 | 10 | 20 | 50 | 100 |
|---|---|---|---|---|---|---|---|
| Mean area | 54.433 | 97.233 | 305.3 | 583.133 | 1108.33 | 2715.433 | 5216.733 |
| SD ± | 0.83 | 1.81 | 2.1 | 2.57 | 3.01 | 2.8 | 3.45 |
| RSD% | 1.54 | 1.86 | 0.69 | 0.44 | 0.27 | 0.1 | 0.06 |
| $R^2$ | 0.9996 | | | | | | |

**Table 1**: Calibration curve parameters for amoxicillin, clarithromycin and esomeprazole (n=3)

Other important parameters like Limit of detection (LOD) and limit of quantification for three drugs were determined on the basis of linearity data. LOD and LOQ for amoxicillin, clarithromycin and esomeprazole were determined in ratio of 3:1 and 10:1 by using the equation (1) and (2). Limit of detection for amoxicillin, clarithromycin and esomeprazole was found to be 0.26169 µg/mL, 8.976 µg/mL and 0.13157 µg/mL respectively. Limit of quantification was 0.79302 µg/mL, 27.200 µg/mL and 0.39872 µg/mL for amoxicillin, clarithromycin and esomeprazole respectively as shown in Table 2. Moreover, accuracy and precision for amoxicillin, clarithromycin and esomeprazole at different concentrations was determined in triplicate injections. Percentage accuracy and relative standard deviation (RSD) for both intra and inter day precision was determined and the obtained results was found to be <2%. Further, percent accuracy was in range of 98-102% for amoxicillin, clarithromycin and esomeprazole. These values of accuracy and precision were within limits, showing that the developed method was accurate and precise. Results obtained from accuracy, Interday precision and intraday precision are shown in Table 3. All the obtained results are close in similarity to the already published literature [15,18,19].

| Drug | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|
| Amoxicillin | 0.26 | 0.79 |
| Clarithromycin | 8.97 | 27.2 |
| Esomeprazole | 0.13 | 0.39 |

**Table 2**: Results of LOD and LOQ

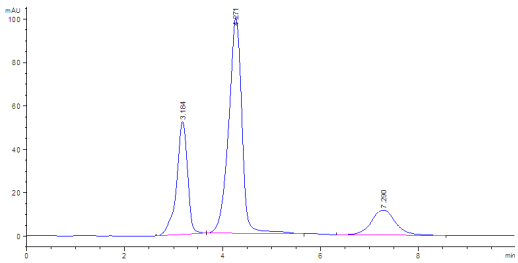


**Figure 2**: Chromatogram of sample containing three drugs amoxicillin (peak at 3.1 min), esomeprazole (peak at 4.2 min) and clarithromycin (peak at 7.2 min)

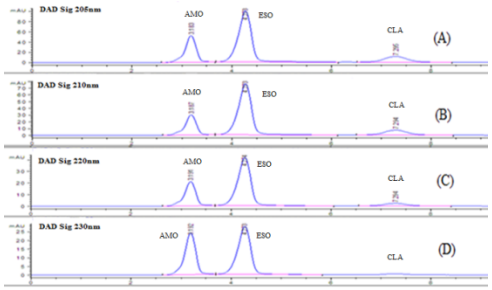


**Figure 3**: Chromatograms of three drugs at different wavelength (A) at 205 nm, (B) at 210 nm, (C) at 220 nm, (D) at 230 nm

| Actual concentrations (µg/mL) | Mean area and RSD (%) | | | | Accuracy (%) |
|---|---|---|---|---|---|
| | Intraday precision | | Inter day precision | | |
| | Area | RSD (%) | Area | RSD (%) | |
| **Amoxicillin** | | | | | |
| 5 | 233.433 | 1.95 | 232.933 | 1.13 | 99.76 |
| 50 | 1447.333 | 0.66 | 1440.733 | 0.6 | 101.61 |
| 100 | 2676.7 | 0.24 | 2683.766 | 0.29 | 99.05 |
| **Clarithromycin** | | | | | |
| 50 | 76.8 | 1.69 | 73.833 | 1.95 | 100.59 |
| 100 | 354.5 | 0.8 | 354.666 | 0.88 | 99.85 |
| 1000 | 708.06 | 0.46 | 703.466 | 0.69 | 100.99 |
| **Esomeprazole** | | | | | |
| 5 | 305.733 | 0.8 | 306.5 | 1.18 | 99.64 |
| 50 | 2714.666 | 0.15 | 2715.13 | 0.19 | 100.46 |
| 100 | 5215.766 | 0.14 | 5212.766 | 0.09 | 99.33 |

**Table 3**: Intra and inter day precision and accuracy data for amoxicillin, clarithromycin and esomeprazole samples (n=3)

## Recovery and Stability studies

Another important parameter for example recovery studies were performed by standard addition method (SAM) in which a known amount of analyte was added to pre analyzed samples at levels of 50, 100 and 150% in triplicate injections for amoxicillin, clarithromycin and esomeprazole. Final

concentrations were 30, 40 and 50 µg/mL for amoxicillin and esomeprazole, while for clarithromycin it was 750, 1000 and 1250 µg/mL. The percent relative standard deviations were less than 2% and the data are summarized in Table 4. Additionally, robustness study was also performed. Robustness has a vital role in development and validation of HPLC method. Developed method should remain unaffected to slight changes in experimental conditions. Robustness of amoxicillin, clarithromycin and esomeprazole was performed by deliberately making small changes in flow rate, organic composition of mobile phase ( ± 2%) and pH of mobile phase ( ± 0.2), all samples were injected in triplicate. Relative standard deviations of peak area, symmetry and retention time were calculated and found to be <2% which shows that the developed method was robust to slight changes in experimental conditions. The obtained results from robustness study were summarized in Table 5 and were found in good relation to the reported results. Stability of amoxicillin, clarithromycin and esomeprazole was evaluated by comparison of standard solutions and test solutions. The solutions of three drugs were stored at room temperature without protection from light and then tested after 24, 48 and 72 hours. Responses for all the three drugs are summarized in Table 6 as assessed by comparing with freshly prepared solutions. The results of stability studies showed that solutions were stable for 72 hours at room temperature.

| Recovery level (%) | Amt. Taken (µg/mL) | Amt. Added (µg/mL) | Total (µg/mL) | Amount Recovered (%) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|
| **Recovery of amoxicillin** | | | | | | |
| 50 | 20 | 10 | 30 | 29.79 | 99.3 | 0.936 |
| 100 | 20 | 20 | 40 | 39.69 | 99.24 | |
| 150 | 20 | 30 | 50 | 50.5 | 101 | |
| **Recovery of clarithromycin** | | | | | | |
| 50 | 500 | 250 | 750 | 755.7 | 100.76 | 0.499 |
| 100 | 500 | 500 | 100 | 1007.06 | 100.7 | |
| 150 | 500 | 750 | 1250 | 1260.45 | 100.83 | |
| **Recovery of esomeprazole** | | | | | | |
| 50 | 20 | 10 | 30 | 30.18 | 100.61 | 0.33 |
| 100 | 20 | 20 | 40 | 40.06 | 100.15 | |
| 150 | 20 | 30 | 50 | 51.03 | 102.06 | |

**Table 4**: Recovery studies of amoxicillin, clarithromycin and esomeprazole (n=3)

| Condition | Parameter | | | | | |
|---|---|---|---|---|---|---|
| | Peak area | %RSD | Retention time (min) | %RSD | Symmetry | %RSD |
| **Mobile Phase Ratio** | | | | | | |
| AMO | 62:38:00 | 1452.9 | 0.26 | 3.12 | 0.25 | 0.8 | 0.37 |
| CLA | | 352.9 | 1.1 | 7.19 | 0.89 | 0.51 | 1.72 |
| ESO | | 2729.5 | 0.66 | 4.22 | 0.19 | 0.8 | 0.43 |
| AMO | 60:40:00 | 1454.5 | 0.94 | 3.1 | 0.06 | 0.91 | 0.16 |
| CLA | | 354.5 | 1.14 | 7.2 | 0.04 | 0.56 | 1.68 |
| ESO | | 2724.3 | 0.6 | 4.21 | 0.22 | 0.92 | 1.1 |
| AMO | 52:42:00 | 1448.5 | 0.49 | 3.13 | 0.99 | 0.9 | 0.68 |
| CLA | | 345.5 | 0.66 | 7.2 | 0.37 | 0.5 | 1.23 |
| ESO | | 2732.2 | 0.47 | 4.17 | 1.12 | 0.91 | 0.75 |
| **Flow Rate mL/min** | | | | | | |
| AMO | 0.98 | 1446.4 | 0.639 | 3.15 | 0.27 | 0.9 | 0.27 |
| CLA | | 357.26 | 1.38 | 7.18 | 1.28 | 0.58 | 1.1 |
| ESO | | 2719.1 | 0.13 | 4.19 | 0.22 | 0.91 | 0.66 |
| AMO | 1 | 1447.7 | 0.64 | 3.15 | 1.83 | 0.91 | 0.66 |
| CLA | | 351.1 | 1.6 | 7.15 | 0.8. | 0.51 | 1.18 |
| ESO | | 2726 | 0.49 | 4.22 | 0.17 | 0.93 | 0.89 |
| AMO | 0.96 | 1451.1 | 0.34 | 3.15 | 1.77 | 0.9 | 0.87 |
| CLA | | 354.43 | 1.72 | 7.11 | 0.18 | 0.51 | 1.54 |
| ESO | | 2751.1 | 0.18 | 4.18 | 1.03 | 0.92 | 0.38 |
| **pH of Mobile Phase** | | | | | | |
| AMO | 4.8 | 1453.9 | 1.01 | 3.12 | 1.27 | 0.9 | 0.68 |
| CLA | | 352.5 | 1.57 | 7.2 | 0.55 | 0.5 | 1.42 |
| ESO | | 2726.2 | 0.42 | 4.16 | 1.28 | 0.8 | 0.77 |
| AMO | 5 | 1561.5 | 0.58 | 3.19 | 0.56 | 0.91 | 0.72 |
| CLA | | 360.23 | 1.86 | 7.2 | 0.48 | 0.51 | 1.29 |
| ESO | | 2751 | 0.4 | 4.2 | 0.59 | 0.81 | 0.76 |
| AMO | 5.2 | 1458.2 | 0.33 | 3.13 | 1.13 | 0.81 | 0.76 |
| CLA | | 358.41 | 1.34 | 7.23 | 0.49 | 0.51 | 1.21 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ESO | 2738.2 | 0.76 | 4.13 | 0.85 | 0.91 | 0.68 |

**Table 5**: Robustness of amoxicillin, clarithromycin and esomeprazole in different conditions (n=3)

| Solution stability of amoxicillin, clarithromycin and esomeprazole | | | | |
|---|---|---|---|---|
| **Drug** | **Initial assay %** | **After 24 h** | **After 48 h** | **After 72 h** |
| Amoxicillin | 99.76 | 99.81 | 99.35 | 98.07 |
| Clarithromycin | 99.63 | 99.68 | 98.703 | 97.78 |
| Esomeprazole | 102.5 | 102.46 | 99.5 | 99.405 |

**Table 6**: Stability studies of amoxicillin, clarithromycin and esomeprazole

## Pharmacokinetic study

Pharmacokinetic studies are always important in every combination therapy; therefore it was considered an important parameter for the present study. In this connection oral doses of all the three drugs were calculated according to the body weight and administered by oral gavage in a single dose. The oral dose for amoxicillin, clarithromycin and esomeprazole was 50 mg/kg, 15 mg/kg and 0.7 mg/kg. Single mice was used for one time blood sample that were collected at predetermine time interval, approximately after 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours and 24 hours. All the blood samples were then centrifuged at 13000 rpm for 10 minutes and plasma was separated. Different pharmacokinetic parameters i.e. peak plasma concentration ($C_{max}$) and time to reach maximum concentration ($T_{max}$) were calculated according to tapezodal method [18]. Moreover, Area under plasma concentration time curve ($AUC_{0-\infty}$) was calculated as $AUC_{0-24}+(C_{last}/K_e)$. Whereas, half-life (t1/2), elimination rate constant ($K_e$) were calculated by using the following formula.

$t1/2=0.693/K_e$ (3)

Results obtained from all the pharmacokinetics parameters are presented in Table 7. Moreover, plasma concentration versus time curves for amoxicillin, clarithromycin and esomeprazole were shown in Figure 4. These pharmacokinetic findings showed that there are no drug-drug interactions in such combinations of different drugs. Furthermore, the obtained results also suggest that similar combination will be highly useful in the treatment of *H. pylori*. The reported results revealed that the developed and validated RP-HPLC method for simultaneous determination of amoxicillin, clarithromycin and esomeprazole could be used potentially for routine quality control analyses in pharmaceutical industries.

| Pharmacokinetic studies of amoxicillin, clarithromycin and esomeprazole | | | |
|---|---|---|---|
| **Parameters** | **Amoxicillin (p.o)** | **Clarithromycin (p.o)** | **Esomeprazole (p.o)** |
| $T_{max}$/h | 2 | 4 | 2 |
| $C_{max}$ (µg/mL) | 1042.17 ± 4.0 | 218.67 ± 5.3 | 18.97 ± 3.6 |
| $C_{min}$ (µg/mL) | 46.99 ± 3.9 | 38.98 ± 4.3 | 1.26 ± 0.17 |
| $K_e$ | 0.93 ± 1.2 | 0.20 ± 2.1 | 0.30 ± 2.5 |
| t1/2/h | 0.73 ± 2.3 | 3.3 ± 3.2 | 2.2 ± 3.1 |
| $AUC_{0-24}$ (µgh/mL) | 6612.00 ± 4.3 | 2073.96 ± 3.4 | 122.04 ± 3.2 |
| $AUC_{0-\infty}$ (µgh/mL) | 6661.06 ± 3.5 | 2261.81 ± 5.3 | 126.15 ± 4.5 |

**Table 7**: Pharmacokinetic parameters of amoxicillin, clarithromycin and esomeprazole after oral administration

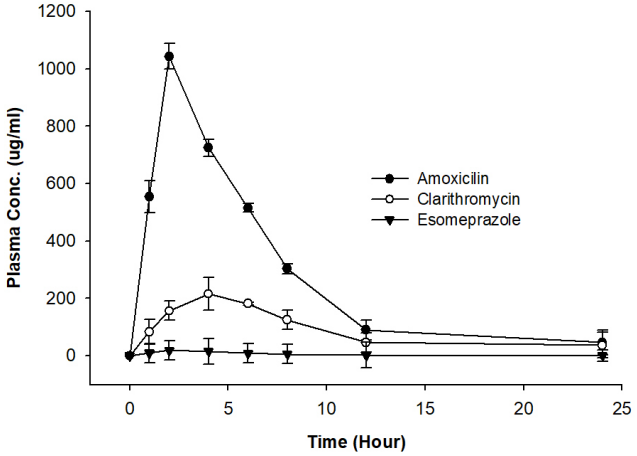


**Figure 4**: Plasma concentration time curve for amoxicillin, clarithromycin and esomeprazole after oral administration as a single dose

## CONCLUSION

An isocratic RP-HPLC method for simultaneous determination of amoxicillin, clarithromycin and esomeprazole has been developed and validated. Furthermore, statistical analysis of the obtained results showed high accuracy and good precision. It can be concluded that amoxicillin, clarithromycin and esomeprazole could be separated using reverse phase C18 column with buffer and methanol (60:40 v/v) as mobile phase. The linear calibration curve could be obtained with percent accuracy in the range of 98-102% for amoxicillin, clarithromycin and esomeprazole. The results showed that amoxicillin, clarithromycin and esomeprazole could be simultaneously analyzed in a solution as well as in plasma.

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## AUTHORS' CONTRIBUTIONS

All authors equally contributed to this research work and approved the final manuscript.

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## AVAILABILITY OF DATA AND MATERIALS

Not applicable

## COMPETING INTERESTS

All the authors declare that they have no competing interests.

## REFERENCES

1. Salih BA. Helicobacter pylori infection in developing countries: the burden for how long? Saudi J Gastroenterol. 2009;15(3):201-207.
2. Kusters JG, Van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. Clin Microbiol Rev. 2006;19(3):449-490.
3. Tulassay Z, Stolte M, Sjolund M, Engstrand L, Butruk E, Malfertheiner P, et al. Effect of esomeprazole triple therapy on eradication rates of Helicobacter pylori, gastric ulcer healing and prevention of relapse in gastric ulcer patients. Eur J Gastroenterol Hepatol. 2008;20(6):526-536.
4. Maddux MS. Effects of ß-Lactamase-Mediated Antimicrobial Resistance: The Role of ß-Lactamase Inhibitors. Pharmacotherapy. 1991;11(2(Pt2)):40S-50S.
5. Nilsson-Ehle I, Fellner H, Hedstrom SA, Nilsson-Ehle P, Sjovall J. Pharmacokinetics of clavulanic acid, given in combination with amoxycillin, in volunteers. J Antimicrobial Chemotherapy. 1985;16(4): 491-498.
6. Todd PA, Benfield P. Amoxicillin/clavulanic acid. An update of its antibacterial activity, pharmacokinetic properties and therapeutic use. Drugs. 1990;39(2):264-307.
7. Dowell SF, Butler JC, Giebink GS, Jacobs MR, Jernigan D, Musher DM, et al. Acute otitis media: management and surveillance in an era of pneumococcal resistance-a report from the Drug-resistant Streptococcus pneumoniae Therapeutic Working Group. Pediatr Infect Dis J. 1999;18(1):1-9.
8. Adam D, Glaser-Caldow E, Wachter J, Brueckner OJ, Hein J, Kroemer B, et al. Comparative efficacy of clarithromycin modified-release and clarithromycin immediate-release formulations in the treatment of lower respiratory tract infection. Clin Ther. 2001;23(4):585-595.
9. Zuckerman JM. Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. Infect Dis Clin North Am. 2004;18(3): 621-649.
10. Goderska K, Pena SA, Alarcon T. Helicobacter pylori treatment: antibiotics or probiotics. Appl Microbiol Biotechnol. 2018;102(1):1-7.
11. Khalil MT, Usman M, Khan GM, Awan SB, Bibi H, Siddiqua A. HPLC method development and validation for the estimation of esomeprazole in bulk and pharmaceutical dosage form. Int J Drug Dev Res. 2012;4(4):252-256.
12. Rele RV, Mali RN. Simultaneous determination of amoxicillin trihydrate and bromhexine hydrochloride in pharmaceutical dosage by reverse phase high performance liquid chromatography. Der Pharma Chem. 2013;5(1):273-278.
13. Li W, Jia H, and Zhao K. Determination of clarithromycin in rat plasma by HPLC-UV method with pre-column derivatization. Talanta. 2007;71(1):385-390.
14. Jiang Y, Wang J, Li H, Wang Y, Gu J. Determination of clarithromycin in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry. J Pharm Biomed Anal. 2007;43(4): 1460-1464.
15. Onal A, Oztunc A. Development and validation of high performance liquid chromatographic method for the determination of esomeprazole in tablets. J Food Drug Anal. 2006;14(1):12.
16. Salem H, Riad SM, Rezk MR, Ahmed K. Simultaneous determination of omeprazole, tinidazole and clarithromycin in bulk powder and helicure tablets by HPLC. J Chromatogr Sep Tech. 2014;5(2):1-5.
17. Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles of young scientists. 2011;2(1):21.
18. Chiou WL. Critical evaluation of the potential error in pharmacokinetic studies of using the linear trapezoidal rule method for the calculation of the area under the plasma level-time curve. J Pharmacokinetics Biopharmaceutics. 1978;6(6):539-546.
19. Jain DK, Jain N, Charde R, Jain N. The RP-HPLC method for simultaneous estimation of esomeprazole and naproxen in binary combination. Pharm Methods. 2011;2(3):167-172.