Multi-analyte HPLC–DAD Method for Concurrent Analysis of Six Antimicrobials and Three Proton Pump Inhibitors Frequently used in Management of Helicobacter pylori Infection: Application to Simulated Intestinal Fluid Samples

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Abstract
The present work deals with the optimization, validation and application of a versatile HPLC–DAD method for concurrent estimation of nine antimicrobials and proton pump inhibitors, namely amoxicillin (AMX), doxycycline (DOX), furazolidone (FRZ), lansoprazole (LNS), levofloxacin (LVF), metronidazole (MTZ), omeprazole (OMZ), pantoprazole (PNZ) and tinidazole (TNZ). The selected nine drugs are frequently included in various treatment regimens of Helicobacter pylori infection. Successful separation was accomplished using the analytical column Agilent Zorbax Eclipse plus-C18 (250 × 4.6 mm, 5 µm particle size) and a mobile phase prepared from phosphate buffer pH 5 and acetonitrile pumped at a flow rate 1 mL/min using a gradient elution program. The gradient elution started with buffer/acetonitrile ratio 90:10, then it was altered in 15 min to reach 40:60 by volume. Quantification of the analytes was based on measuring peak areas of AMX at 230 nm, LVF, LNS and PNZ at 290 nm, OMZ at 300 nm, MTZ and TNZ at 320 nm, and DOX and FRZ at 360 nm. The separated compounds eluted at retention times 5.68, 6.43, 7.82, 8.84, 9.42, 10.75, 12.82, 13.74 and 14.90 min for AMX, MTZ, LVF, TNZ, DOX, FRZ, OMZ, PNZ and LNS respectively. Validation of the proposed HPLC procedure was carefully studied according to the ICH items: ranges, precision, accuracy, linearity, robustness and limits of detection and quantitation. The linear dynamic ranges were 5–100, 5–50, 2–40, 10–100, 5–50, 5–50, 2.5–30, 3–30 and 2–30 µg/mL for AMX, MTZ, LVF, TNZ, DOX, FRZ, OMZ, PNZ and LNS, respectively with correlation coefficients > 0.9993. Application fields of the validated method included analysis of laboratory-prepared binary dosage forms along with analysis of several ternary mixtures in spiked simulated intestinal fluid.

Keywords  HPLC–DAD · Antimicrobial · Proton pump inhibitor · Helicobacter pylori · Simulated intestinal fluid

Introduction
Helicobacter pylori is a Gram-negative bacterium that causes both non-malignant and malignant gastro-duodenal diseases, such as dyspepsia, chronic gastritis, peptic ulcers, and gastric cancer. It is estimated that H. pylori infection spreads in about half of the world’s population with higher occurrence in developing countries [1]. Numerous reported guidelines and regimens for H. pylori treatment have been published and clinically investigated. Triple therapy is still the most frequently used treatment which includes a pair of antibiotics with a proton pump inhibitor (PPI). Although there are different treatments, the infection rate is still increasing worldwide. The challenge of treatment failure is greatly due to the resistance of H. pylori to antibiotics and due to their side effects. Choices after initial eradication failure include specific personalized therapy (selecting antibiotic combinations based on antibiotic susceptibility tests) and/or quadruple therapy where a bismuth salt (e.g., bismuth subcitrate) is added as the fourth component in the treatment plan [2, 3].

Due to the surge in the occurrence of H. pylori resistance to antibiotics, triple therapy containing clarithromycin is no longer a reliable treatment for H. pylori, particularly...
in areas where resistance to this antibiotic is higher than 20% [3–5]. Therefore, clarithromycin is not included in this research work. On the other hand, amoxicillin-resistant *H. pylori* strains have hardly been detected; therefore, amoxicillin is still regularly used in most treatments for *H. pylori* infection [6]. Other antibiotics from different classes have been frequently used, such as doxycycline, furazolidone and levofloxacain [7–13]. In addition, the nitroimidazole antimicrobials metronidazole and tinidazole are often used as well in different eradication plans [3, 14–17].

Analyses of different mixtures containing these antimicrobials were described in several articles. The well-known binary mixture of amoxicillin and metronidazole was assayed using spectrophotometric methods [18], HPLC with UV or photodiode array detection [19, 20] and hydrophilic interaction chromatography (HILIC) followed by mass spectrometric (MS) detection [21]. Another binary fixed-dose combination available in the international markets contains doxycycline and tinidazole. Simultaneous estimation of these two drugs was carried out using spectrophotometric methods manipulating ratio spectra [24], Fourier-transform infrared (FTIR) spectroscopy [25], TCL–densitometry [26] and HPLC with online UV wavelength switching detection [27]. Other clarithromycin-containing ternary mixtures for *Helicobacter* infection treatment were determined using TCL–densitometry [28] and chemometrics-assisted HPLC [29].

The triple mixture of doxycycline, tinidazole and omeprazole (sometimes together with clarithromycin) attracted some attention from several Egyptian research groups. Suggested methods of analysis included smart spectrophotometric procedures utilizing zero order, ratio or derivative spectra [30] and RP-HPLC with UV detection [31–33]. Additionally, HPLC with UV detection method was suggested for the simultaneous quantitation of amoxicillin, metronidazole, and pantoprazole in human plasma [34]. Moreover, HPLC–DAD was adopted for determination of mixture of amoxicillin, metronidazole and rabeprazole sodium and the developed method was applied to simulated intestinal fluid spiked with the three drugs [35]. Recently, concurrent analysis of amoxicillin, lansoprazole and levofloxacin in pharmaceuticals was accomplished by means of HPLC with UV–Vis detector [36] and ratio derivative spectrophotometry [37].

Development of multi-analyte “broad spectrum” chromatographic methods for separation and simultaneous determination of several structurally or pharmacologically related drugs is one of the modern trends in drug analysis. These versatile methods are economic and time-effective hence can be applied for assay of several drug mixtures in quality control laboratories. Different groups of related pharmaceutical compounds have been assayed using such methods [38–42]. The objective of the present work is to design and evaluate a multi-analyte and versatile HPLC–DAD method that serves in various applications. Analytes include six antimicrobials commonly used for *H. pylori* eradication, namely amoxicillin, doxycycline, furazolidone, levofloxacin, metronidazole and tinidazole in addition to threes PPIs: omeprazole, pantoprazole and lansoprazole. Chemical structures of the nine inspected drugs are depicted in Fig. 1. To the best of our knowledge, there is no single chromatographic method reported to cover the simultaneous analysis of the cited multi-class drugs. Moreover, combinations of levofloxacin and furazolidone with any of the three PPIs have not been investigated yet in any analytical report. The proposed method was applied to fulfill several targets, first: construction of a multi-analyte method for separation of nine different drugs used in *H. pylori* management; second: estimation of spiked simulated intestinal fluid samples where three different ternary mixtures were investigated; third: assay of pharmaceutical dosage forms.

### Materials and Methods

#### Instrumentation

HPLC–DAD system consisted of Agilent 1200 series (auto-injector, quaternary pump, vacuum degasser and diode array multiple wavelength detector) connected to a computer loaded with Agilent ChemStation Software (Agilent Technologies, Santa Clara, CA, USA). Measurements of pH were made using Crison pH meter, S.A. (Barcelona, Spain). Celulose nitrate membrane filters (0.45 µm) (Sartorius Stedim Biotech GmbH 37,070 Goettingen, Germany) were used.

#### Materials and Reagents

Amoxicillin trihydrate (AMX), Doxycycline hydrochloride (DOX), Furazolidone (FRZ), Lansoprazole (LNS), Levofloxacain (LVF), Metronidazole (MTZ), Omeprazole (OMZ), Pantoprazole sodium (PNZ) and Tinidazole (TNZ) were kindly supplied by Pharco Pharmaceuticals Company, Alexandria, Egypt. Analytical grade of orthophosphoric acid, potassium dihydrogen phosphate, sodium hydroxide and hydrochloric acid were purchased from El-Nasr Chemical Industry Company, Cairo. Anhydrous sodium dihydrogen phosphate was supplied by Loba Chemie PVT. LTD., Mumbai, India. Acetonitrile and methanol (HPLC grade) were obtained from Avantor performance materials, Poland S.A. Pancreatin (obtained from porcine pancreas powder) was manufactured by Deyang Sinozyme Pharmaceutical Co, Ltd, Xiaohan, Guanhuan, Sichuan Province, China. High-purity distilled water was used. The pharmaceutical
formulations assayed in the study were laboratory-made capsules containing 250 mg AMX and 200 mg MTZ per capsule and laboratory-made tablets containing 100 mg DOX and 600 mg TNZ per tablet. The excipients (starch, lactose, talc and magnesium stearate) were added.

**Chromatographic Conditions**

Agilent Zorbax Eclipse plus-C18 (250 × 4.6 mm, 5 μm particle size) column was utilized. Mobile phase consisting of phosphate buffer pH 5 and acetonitrile was employed in gradient elution starting with the ratio 90:10 by volume. This ratio was linearly changed in 15 min to reach 40:60 by volume, and it was kept for 1 min. Before starting the next run, the program was returned to the initial ratio (phosphate buffer and acetonitrile, 90:10) and the analytical column was reconditioned for 3 min. The phosphate buffer was composed of 0.025 M NaH₂PO₄ which was prepared by dissolving 0.88 g NaH₂PO₄ in 250 mL distilled water and adjusting to pH 5.0 using orthophosphoric acid. The mobile phase was filtered prior to use and pumped at flow rate 1 mL min⁻¹. Injection volume was 20 μL. Column temperature was kept at 25 °C. Chromatograms were recorded by the diode array detector (DAD) at the wavelengths 230, 290, 300, 320 and 360 nm.

**Preparation of Stock Standard Solutions**

Stock standard solutions of AMX and OMZ (500 μg mL⁻¹) were separately prepared in methanol. Stock standard solutions of DOX and TNZ (1000 μg mL⁻¹) and of LVF, MTZ and PNZ (500 μg mL⁻¹) were separately prepared in distilled
water. Finally, stock standard solution of FRZ (250 µg mL⁻¹) and of LNS (500 µg mL⁻¹) was separately prepared in acetonitrile. All stock solutions were stored and refrigerated at 4 °C.

**Method Validation**

ICH guidelines on validation of analytical procedures [43] were followed for the purpose of validation and testing performance of the developed method.

**Linearity and Ranges**

The method linearity was assessed by examining various concentrations of each of the nine drugs. Dilution of serial volumes of the stock solutions of the nine drugs was done with HPLC-grade methanol to reach the concentration ranges 5–100, 5–50, 2–40, 10–100, 5–50, 2.5–30, 3–30 and 2–30 µg mL⁻¹ for AMX, MTZ, LVF, TNZ, DOX, FRZ, OMZ, PNZ and LNS, respectively. Triplicate 20 µL injections were chromatographed for each concentration. The peak areas of AMX (at 230 nm), LVF, LNS and PNZ (at 290 nm), OMZ (at 300 nm), MTZ and TNZ at (320 nm) and DOX and FRZ (at 360 nm) were plotted against the corresponding concentrations to construct the calibration graphs, and regression equation for each drug was computed. In addition, other statistical parameters, such as standard deviations of the intercept (Sₐ), slope (Sₜₘ), residuals (Sᵧₓᵢₑ), correlation and determination coefficients, were computed.

**Limits of Detection and Quantitation**

The limits of detection (LOD) and limits of quantitation (LOQ) were calculated as guided by the ICH based on signal-to-noise ratio method. LOD is defined as the concentration level that is pertinent to a signal-to-noise ratio of 3:1, while for LOQ, the ratio is 10:1.

**Accuracy and Precision**

Three concentration levels for each drug across its working range were selected for validating accuracy and precision of the optimized method. The accuracy and intra-day precision were evaluated at the three concentration levels for every analyte using triplicate measurements of every concentration in one day. Likewise, the inter-day precision was examined by analyzing the same prepared concentrations with triplicate measurements performed on three different days. Found concentrations were calculated using the corresponding regression equations. Precision and accuracy of measurement were demonstrated by the calculated percentage relative standard deviation (RSD %) and percentage relative error (Eᵢ, %) values.

**Robustness**

Robustness was inspected by performing minor alterations in acetonitrile content in the mobile phase (± 2%), flow rate (± 0.05 mL min⁻¹), pH of the buffer (± 0.2) and detection wavelengths (± 2 nm) and monitoring the chromatographic behavior of a standard mixture of the nine drugs. Retention times, peak areas and resolution values were recorded. The 3 responses (optimized, higher and lower change) were exploited for calculation of the average response ± SD (n = 3) for peak areas, retention times and resolutions for all analytes upon testing each parameter. Furthermore, RSD % values were calculated for all measured responses.

**Application in Assay of Laboratory-Made Pharmaceutical Preparations**

Two laboratory-made preparations of mixture I (AMX 250 mg and MTZ 200 mg per capsule) and mixture II (DOX 100 mg and TNZ 600 mg per tablet) were prepared and analyzed by the proposed HPLC method. Laboratory-made capsules (20 capsules) were carefully emptied, weighed and mixed; and 20 laboratory-made tablets were weighed and finely powdered. For each preparation, an accurate weight of the homogenized powder equivalent to the average weight per capsule or tablet was mixed and stirred with 60 mL methanol for 10 min then filtered into a 100-mL volumetric flask. Two 10 mL portions of methanol were added to each residue, washings were added to the filtrate and the solution was diluted to volume with methanol to obtain final concentrations 2500 µg mL⁻¹ AMX and 2000 µg mL⁻¹ MTZ (for mixture I) and 1000 µg mL⁻¹ DOX and 6000 µg mL⁻¹ TNZ (for mixture II). Accurate volumes from these stock sample solutions were diluted with methanol to reach concentration levels within the formerly described ranges and the diluted sample solutions were chromatographed using the developed method. Recoveries were calculated from similarly treated standard solutions.

**Application in Analysis of Spiked Simulated Intestinal Fluid Samples**

The artificial intestinal fluid was prepared as described in both the British and United States Pharmacopeias [44, 45]. A weight of 6.8 g of dihydrogen potassium phosphate was dissolved in 250 mL distilled water and mixed with 77 mL of 0.2 M sodium hydroxide solution and 500 mL of distilled water. A weight of 10 g pancreatin was added and the pH
was adjusted to 6.8 ± 0.1 with either NaOH or HCl (0.2 M solutions). Finally, the volume was made up to 1000 mL with distilled water.

Aliquots from the stock solutions of the nine drugs were mixed and diluted with the simulated intestinal fluid to prepare ternary mixtures containing drug concentrations within their described ranges. The ternary mixtures were prepared in concentration levels pertinent to their anticipated ratios when the three drugs are co-administered. For mixture I, the three drugs AMX, MTZ and PNZ are co-administered in doses 1 g, 500 mg and 40 mg respectively [46]. Therefore, they are prepared in the following concentrations 100 µg/mL AMX, 50 µg/mL MTZ and 4 µg/mL PNZ. In mixture II, the three drugs LVF, FRZ and LNS are given in doses 500 mg, 100 mg and 30 mg respectively [47], hence they are prepared in the concentrations 40 µg/mL LVF, 8 µg/mL FRZ and 2.4 µg/mL LNS. Finally, in mixture III, the three drugs TNZ, DOX and OMZ are prescribed in doses 500 mg, 50 mg and 20 mg respectively [17]; thus, they are prepared in the concentrations 100 µg/mL TNZ, 10 µg/mL DOX and 4 mg OMZ. The prepared mixtures were filtered through 0.45 µm cellulose nitrate membrane filters and the general procedure was then applied. Recovered concentrations were computed from similarly prepared standard solutions.

Results and Discussion

Optimization of the Separation Method

An HPLC–DAD method was proposed for the multi-class separation and simultaneous quantitation of nine drugs frequently administered for treatment of *H. pylori* infection, namely, AMX, MTZ, LVF, TNZ, DOX, FRZ, OMZ, PNZ and LNS. The nine compounds were effectively separated on an Agilent Zorbax Eclipse plus-C18 (250 × 4.6 mm, 5 µm particle size) column. The multiple wavelength detector was very important to ensure measurement of each analyte at its optimal wavelength.

The achievement of acceptable resolution and peak shape within suitable run time is the main target during LC method development. Accordingly, several experiments were performed to optimize both the stationary and mobile phases. Analytical columns tested in this study were Agilent Zorbax SB-C8 Stable Bond column (4.6 × 250 mm, 5 µm particle size), Agilent Zorbax Eclipse plus-C18 column (4.6 × 250 mm, 5 µm particle size), Waters Symmetry C8 (3.9 × 150 mm, 5 µm particle size) and Waters Symmetry C18 (3.9 × 150 mm, 5 µm particle size). Different mobile phases containing phosphate buffer with either acetonitrile or methanol pumped using different gradient programs were tested with each column. The Agilent Zorbax Eclipse plus-C18 column was found ideal because it resulted in the best separation between the eluting peaks in a moderately short run time, accordingly, it was employed as the working column for the current study.

Mobile phases of various composition were evaluated using different aqueous phases and organic modifiers adjusted at various pH values. Good separation was attained with a mobile phase consisting of phosphate buffer pH 5 and acetonitrile in gradient elution starting with the ratio 90:10 by volume, then it was changed linearly in 15 min to reach the ratio 40:60. The effect of mobile phase pH was inspected within the range 3–6 and the best chromatographic picture was obtained at pH 5. At pH 3, DOX and FRZ peaks were overlapped together, while upon using pH 4, tailed and asymmetric OMZ, PNZ and LNS peaks were observed. In contrast, buffer pH 6 produced good separation between peaks; nevertheless, the run time was longer; thus, pH 5 was chosen as the best compromise. Methanol as an organic modifier was tried with phosphate buffer at pH 5 in different gradient elution programs, but most drug peaks were overlapped and not enough separated, so acetonitrile with phosphate buffer were found the best mobile phase combination for assay of this complex mixture. Additionally, phosphate buffer and acetonitrile were examined in different gradient elutions, and the best separation picture with the shortest run time was obtained using the previously mentioned program. A flow rate of 1 mL/min was found ideal regarding runtime, peak asymmetry and column pressure. Finally, column temperature was adjusted at 25 °C throughout the chromatographic run.

DAD was exploited for quantitation of the analytes based on peak area measurements. This multiple wavelength detector guarantees the advantage of measuring each analyte at its selected wavelength within the same run which improves sensitivity. This is crucial in case of analytes with different absorption features and λ_max values. AMX exhibits an absorption spectrum with maximum absorption at 230 nm; LVF, PNZ and LNS show absorption spectra with maxima at 290 nm, OMZ shows an absorption peak at 300 nm; the nitroimidazoles MTZ and TNZ have absorption spectra peaking at 320 nm, and finally, DOX and FRZ display wide absorption spectra with several peaks of which the highest at 360 nm.

The above-described chromatographic conditions exhibited almost symmetric peaks and excellent resolution between the nine drugs within acceptable run time. Figure 2 displays the separation chromatogram for the nine compounds at 230 nm. Figures 1S–4S in the Supplementary file show chromatograms for the same mixture at the other working wavelengths. AMX, MTZ, LVF, TNZ, DOX,
FRZ, OMZ, PNZ and LNS eluted at retention times 5.68, 6.43, 7.82, 8.84, 9.42, 10.75, 13.74 and 14.90 min, respectively. Resolution ($R_s$) value expresses the degree of separation between successive peaks. A minimum resolution value of 1.5 is a requirement for complete baseline separation between two successive peaks [48, 49]. Calculated resolution values between all peaks in this mixture were at least 3.22 which confirmed excellent separation between the eluting peaks. System suitability metrics were determined for the nine peaks of this mixture and they were found satisfactory [48, 49] (Table 1S in the Supplementary file).

### Method Validation

#### Linearity and Ranges

The recorded peak areas at the specified wavelengths were directly proportional to the drug concentrations. Table 1 assembles the linearity data and statistical parameters for analysis of the nine drugs including concentration ranges, linear regression equations (intercepts and slopes), standard deviations of the intercept ($S_a$), slope ($S_b$), correlation and determination coefficients.

![HPLC chromatogram at 230 nm of a mixture containing 15 g/mL AMX, 15 g/mL MTZ, 6 g/mL LVF, 30 g/mL TNZ, 35 g/mL DOX, 15 g/mL FRZ, 20 g/mL OMZ, 20 g/mL PNZ and 20 g/mL LNS](image)

**Table 1** Analytical parameters for determination of the nine drugs using the proposed HPLC–DAD method

| Parameter          | AMX  | MTZ  | LVF  | TNZ  | DOX  | FRZ  | OMZ  | PNZ  | LNS  |
|--------------------|------|------|------|------|------|------|------|------|------|
| Range (µg/mL)      | 5–100| 5–50 | 2–40 | 10–100| 10–100| 5–50 | 2.5–30| 3–30 | 2–30 |
| Wavelength (nm)    | 230  | 320  | 290  | 320  | 360  | 360  | 300  | 290  | 290  |
| Intercept (a)      | 20.90| −25.91| 37.46| 68.85| 52.63| −38.70| 18.27| 2.44 | 44.05|
| $S_a$              | 13.30| 10.93| 3.35 | 8.21 | 3.92 | 12.86| 14.86| 5.95 | 16.42|
| Slope (b)          | 37.32| 61.01| 75.39| 40.89| 23.18| 81.00| 50.60| 40.63| 83.14|
| $S_b$              | 0.25 | 0.37 | 0.17 | 0.15 | 0.07 | 0.45 | 0.82 | 0.32 | 0.91 |
| RSD% of the slope  | 0.67 | 0.61 | 0.23 | 0.37 | 0.30 | 0.56 | 1.62 | 0.79 | 1.10 |
| Correlation coefficient (r) | 0.99988 | 0.99990 | 0.99998 | 0.99996 | 0.99969 | 0.99990 | 0.99933 | 0.99967 | 0.99970 |
| Determination coefficient ($r^2$) | 0.99976 | 0.99980 | 0.99994 | 0.99994 | 0.99959 | 0.99980 | 0.99866 | 0.99934 | 0.99940 |
| $S_{\delta a}$     | 22.64| 14.96| 6.20 | 11.80| 5.81 | 18.35| 20.67| 8.19 | 23.09|
| LOD (µg/mL)        | 0.72 | 1.19 | 0.39 | 1.90 | 1.21 | 0.48 | 0.62 | 0.80 | 0.57 |
| LOQ (µg/mL)        | 2.41 | 3.96 | 1.32 | 6.35 | 4.03 | 1.60 | 2.07 | 2.67 | 1.90 |

$S_a$ standard deviation of the intercept  
$S_b$ standard deviation of the slope  
$S_{\delta a}$ standard deviation of residuals
Correlation coefficients \( (r > 0.9993) \) and determination coefficient values \( (r^2 > 0.99866) \) confirm good linearity. Additionally, deviation around the slope expressed as RSD\% of the slope \( (S_b \%) \) values was found less than 1.70%.

**Limits of Detection and Quantitation**

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as guided by the ICH signal-to-noise ratio method. Values given in Table 1 ensure that the proposed method is sufficiently sensitive to detect the drugs in concentrations down to 0.39–1.90 µg mL\(^{-1}\).

**Accuracy and Precision**

Table 2 comprises data for assessment of accuracy and precision of the method. The found concentrations together with % recoveries were satisfying with percentage relative standard deviation (RSD \%) and percentage relative error (E\%\) values never exceeding 2\%, thus verifying the high precision and accuracy of the developed method for estimation of the analytes in their bulk form.

**Robustness**

The robustness of an analytical procedure expresses its capability to remain working unaffected by slight but intentional variations in the experimental parameters and reflects its reliability during regular practice. Robustness was examined by performing small variations in acetonitrile content in the mobile phase (±2\%), flow rate (±0.05 mL/min), pH of the buffer (±0.2) and detection wavelengths (±2 nm) and recording the chromatograms of a standard mixture of the 9 drugs. No significant changes were observed in the measured responses (peak area), retention times or resolution between peaks. Table 2S in the Supplementary file shows the effects of the studied variations on the retention times, resolutions and peak areas of the nine studied drugs. Resolution between any two successive peaks was not less than 3.42 which confirms adequate baseline separation between all peaks.

**Stability of Solutions**

Stability of standard stock solutions in different solvents was examined, they were found stable for about one week when kept refrigerated at 4 ± 2 °C. Also, the working solutions prepared in HPLC-grade methanol showed good stability and no chromatographic fluctuations were observed in the time range of 6 h at room temperature (25 ± 2 °C). Peak areas and retention times of all drugs remained unchanged with % RSD values of the measured peak areas did not exceed 2.0% during this period.

**Applications of the Validated Method**

**Determination of Two Binary Laboratory-Prepared Formulations**

Due to the unattainability of the commercial products in the Egyptian market, laboratory-made tablets containing DOX 100 mg and TNZ 600 mg in one tablet, and laboratory-made capsules containing AMX 250 mg and MTZ 200 mg per capsule were prepared and the validated HPLC–DAD procedure was applied for assay of each mixture. Figure 5S in the Supplementary file represents a chromatogram of a mixture containing AMX 25 µg/mL and MTZ 20 µg/mL at 230 nm. Figure 6S in the Supplementary file shows a chromatogram of a mixture containing TNZ 90 µg/mL and DOX 15 µg/mL at 320 nm. The four drugs eluted at their definite retention times and the chromatograms did not contain any surplus peaks for the inactive ingredients. Additionally, to validate method specificity, the added excipients during preparation of the assayed formulations have been similarly extracted and their extract has been analyzed. Chromatograms shown in Figure 7S in the Supplementary file confirm the absence of any extra peaks that may interfere in analysis of the target drugs. Analysis results exposed good accuracy and precision with accepted values of % recoveries, SD, RSD \% and Er \% (Table 3S in the Supplementary file).

**Determination of Three Ternary Mixtures in Simulated Intestinal Fluid**

The instability and degradation of the proton pump inhibitors OMZ, PNZ and LNS in acidic medium have been reported in several previous studies. Accordingly, OMZ, PNZ and LNS are typically targeted to the intestine by formulation as enteric-coated tablets or gastro-resistant pellets to prevent degradation of the drug by gastric acidity [50–54]. The validated HPLC–DAD procedure was practiced to determine three ternary mixtures (AMX, MTZ and PNZ), (LVF, FRZ and LNS) and (TNZ, DOX, and OMZ) in simulated intestinal fluid which is recommended by the international pharmacopeias to mimic the natural intestinal juice [44, 45]. Sample mixture solutions were prepared by mixing accurate volumes of the stock solutions of the drugs, dilution with the simulated intestinal fluid and filtration prior to injection to the analytical column. It is noteworthy to mention that the drugs’ concentrations in the mixture samples are similar to their anticipated ratios when the drugs are co-administered for *H. pylori* infection treatment [17, 46, 47].
Table 2  Precision and accuracy for analysis of the nine drugs in their bulk form using the proposed HPLC–DAD method

| Drug | Type of analysis | Nominal value (μg/mL) | Found ± SDa (μg/mL) | % Recovery | RSD (%) | Er (%) |
|------|------------------|-----------------------|---------------------|------------|---------|-------|
| AMX  | Within-day       | 10                    | 9.98 ± 0.14         | 99.80      | 1.40    | −0.20 |
|      |                   | 50                    | 49.96 ± 0.28        | 99.92      | 0.56    | −0.08 |
|      |                   | 100                   | 99.50 ± 1.19        | 99.50      | 1.20    | −0.50 |
|      | Between-day       | 10                    | 10.00 ± 0.03        | 100.00     | 0.30    | 0.00  |
|      |                   | 50                    | 49.68 ± 0.50        | 99.36      | 1.02    | −0.64 |
|      |                   | 100                   | 100.43 ± 0.08       | 100.43     | 0.08    | 0.43  |
| MTZ  | Within-day       | 10                    | 9.98 ± 0.10         | 99.80      | 1.00    | −0.20 |
|      |                   | 25                    | 24.62 ± 0.81        | 98.48      | 0.32    | −1.52 |
|      |                   | 50                    | 49.34 ± 0.81        | 98.68      | 1.64    | −1.32 |
|      | Between-day       | 10                    | 10.00 ± 0.04        | 100.00     | 0.40    | 0.00  |
|      |                   | 25                    | 25.10 ± 0.02        | 100.40     | 0.07    | 0.40  |
|      |                   | 50                    | 49.63 ± 0.12        | 99.26      | 0.24    | −0.74 |
| LVF  | Within-day       | 6                     | 5.89 ± 0.07         | 98.17      | 1.18    | −1.83 |
|      |                   | 16                    | 16.04 ± 0.15        | 100.25     | 0.94    | 0.25  |
|      |                   | 30                    | 29.67 ± 0.19        | 98.90      | 0.64    | −1.10 |
|      | Between-day       | 6                     | 5.88 ± 0.01         | 98.00      | 0.17    | −2.00 |
|      |                   | 16                    | 15.79 ± 0.10        | 98.69      | 0.63    | −1.31 |
|      |                   | 30                    | 29.55 ± 0.16        | 98.50      | 0.54    | −1.50 |
| TNZ  | Within-day       | 20                    | 20.25 ± 0.02        | 101.25     | 0.10    | 1.25  |
|      |                   | 50                    | 50.14 ± 0.46        | 100.28     | 0.92    | 0.28  |
|      |                   | 100                   | 100.16 ± 0.36       | 100.16     | 0.36    | 0.16  |
|      | Between-day       | 20                    | 19.83 ± 0.18        | 99.15      | 0.91    | −0.85 |
|      |                   | 50                    | 50.81 ± 0.34        | 101.62     | 0.67    | 1.62  |
|      |                   | 100                   | 100.08 ± 0.81       | 100.08     | 0.81    | 0.08  |
| DOX  | Within-day       | 20                    | 19.83 ± 0.18        | 99.15      | 0.91    | −0.85 |
|      |                   | 35                    | 35.02 ± 0.08        | 100.06     | 0.23    | 0.06  |
|      |                   | 75                    | 74.86 ± 0.89        | 99.81      | 1.19    | −0.19 |
|      | Between-day       | 20                    | 20.30 ± 0.03        | 101.50     | 0.15    | 1.50  |
|      |                   | 35                    | 35.10 ± 0.50        | 100.29     | 1.42    | 0.29  |
|      |                   | 75                    | 74.32 ± 0.71        | 99.09      | 0.96    | −0.91 |
| FRZ  | Within-day       | 10                    | 10.05 ± 0.15        | 100.50     | 1.49    | 0.50  |
|      |                   | 20                    | 20.17 ± 0.34        | 100.85     | 1.69    | 0.85  |
|      |                   | 50                    | 49.91 ± 0.06        | 99.82      | 0.12    | −0.18 |
|      | Between-day       | 10                    | 10.18 ± 0.05        | 101.80     | 0.49    | 1.80  |
|      |                   | 20                    | 19.92 ± 0.13        | 99.60      | 0.65    | −0.40 |
|      |                   | 50                    | 50.58 ± 0.11        | 101.16     | 0.22    | 1.16  |
| OMZ  | Within-day       | 10                    | 10.01 ± 0.13        | 100.10     | 1.30    | 0.10  |
|      |                   | 20                    | 19.82 ± 0.09        | 99.10      | 0.45    | −0.90 |
|      |                   | 25                    | 24.88 ± 0.14        | 99.52      | 0.56    | −0.48 |
|      | Between-day       | 10                    | 10.16 ± 0.09        | 101.60     | 0.89    | 1.60  |
|      |                   | 20                    | 19.73 ± 0.16        | 98.65      | 0.81    | −1.35 |
|      |                   | 25                    | 24.60 ± 0.08        | 98.40      | 0.33    | −1.60 |
| PNZ  | Within-day       | 10                    | 9.90 ± 0.17         | 99.00      | 1.71    | −1.00 |
|      |                   | 20                    | 20.15 ± 0.16        | 100.75     | 0.79    | 0.75  |
|      |                   | 25                    | 25.43 ± 0.23        | 101.72     | 0.90    | 1.72  |
|      | Between-day       | 10                    | 9.88 ± 0.13         | 98.80      | 1.32    | −1.20 |
|      |                   | 20                    | 20.08 ± 0.05        | 100.40     | 0.25    | 0.40  |
|      |                   | 25                    | 25.40 ± 0.20        | 101.60     | 0.79    | 1.60  |
Chromatograms of the analyzed mixture solutions confirmed elution of the nine drugs at their specified retention times and no interference peaks were observed. Figures 8S and 9S in the Supplementary file show the chromatograms of analysis of a standard mixture solution of 100 µg mL⁻¹ AMX, 50 µg mL⁻¹ MTZ and 4 µg mL⁻¹ PNZ prepared in methanol at 230 and 290 nm, respectively. Figures 10S and 11S display the separation chromatograms of the same previous mixture spiked in simulated intestinal fluid at the same wavelengths. Figures 12S and 13S in the Supplementary file include the chromatograms of a standard mixture solution of 100 µg mL⁻¹ TNZ, 10 µg mL⁻¹ DOX and 4 µg/mL OMZ prepared in methanol at 300 and 360 nm, respectively. Figures 14S and 15S represent the chromatograms of analysis of the three mixtures in spiked simulated intestinal fluid at the previously mentioned wavelengths. Figure 16S in the Supplementary file comprises a chromatogram of separation of a standard mixture solution of 40 µg mL⁻¹ LVF, 8 µg mL⁻¹ FRZ and 2.4 mg mL⁻¹ LNS prepared in methanol at 290 nm. Finally, Fig. 17S shows the assay chromatogram of the same mixture solution in simulated intestinal fluid at the assigned wavelength.

Peak purity test results obtained from the diode array detector (DAD) verified peaks’ purity and homogeneity in all the analyzed samples. Purity plots for the nine drugs are given in Figure 18S in the Supplementary file. Furthermore, a blank (drug free)-simulated intestinal fluid was analyzed, and its chromatograms at the studied wavelengths displayed no interfering peaks eluting at/or near the retention times of any of the nine analytes (Figures 19S and 20S in the Supplementary file). It is noteworthy to mention that the nearest background peak eluted at retention time 5.16 min just before AMX peak (Figure 9S in the Supplementary file). Resolution between both peaks was found 1.51 which confirmed adequate separation between these two specific peaks. Peak purity results together with the blank chromatograms can be considered proofs for specificity of the method and its successful applicability. Analysis results of the ternary mixture samples presented satisfying accuracy and precision as shown from the recovered concentrations, SD, RSD % and Er % values (Table 3). In general, RSD% and Er % values for analysis of the three mixtures in simulated intestinal fluid did not exceed 3.7%.

### Table 2 (continued)

| Drug | Type of analysis | Nominal value (µg/mL) | Found ± SDa (µg/mL) | % Recovery | RSD (%) | Er (%) |
|------|------------------|-----------------------|---------------------|------------|---------|--------|
| LNS  | Within-day       | 10                    | 10.06 ± 0.06        | 100.60     | 0.60    | 0.60   |
|      |                  | 15                    | 14.80 ± 0.26        | 98.67      | 1.76    | −1.33  |
|      |                  | 20                    | 19.65 ± 0.15        | 98.25      | 0.76    | −1.75  |
|      | Between-day      | 10                    | 10.16 ± 0.02        | 101.60     | 0.20    | 1.60   |
|      |                  | 15                    | 14.87 ± 0.03        | 99.13      | 0.20    | −0.87  |
|      |                  | 20                    | 19.63 ± 0.31        | 98.15      | 1.58    | −1.85  |

aMean ± standard deviation for three determinations

### Table 3

| Nominal value (µg/mL) | Found ± SDa (µg/mL) | RSD (%) b | Er (%) c |
|-----------------------|---------------------|-----------|----------|
| AMX MTZ PNZ           | AMX MTZ PNZ         | AMX MTZ PNZ | AMX MTZ PNZ |
| 100 50 4              | 99.50 ± 1.47        | 4.02 ± 0.07 | 1.48    | 1.04    | −1.72  |
| 100 50 10             | 98.99 ± 1.32        | 10.26 ± 0.03 | 1.33    | 1.10    | −1.01  |
| LVF FRZ LNS           | LVF FRZ LNS         | LVF FRZ LNS | LVF FRZ LNS |
| 40 8 2.4              | 41.49 ± 0.07        | 2.33 ± 0.02 | 0.17    | 2.29    | 0.86   |
| 40 8 9               | 40.55 ± 0.87        | 8.77 ± 0.07 | 2.15    | 1.15    | 0.80   |
| TNZ DOX OMZ          | TNZ DOX OMZ         | TNZ DOX OMZ | TNZ DOX OMZ |
| 100 10 4             | 101.71 ± 0.58       | 3.90 ± 0.01 | 0.57    | 1.22    | 0.26   |
| 100 10 10            | 102.65 ± 0.15       | 9.82 ± 0.10 | 0.15    | 0.72    | 1.02   |

aMean ± standard deviation for three determinations
b% Relative standard deviation
c% Relative error
Conclusion

A specific, robust and versatile HPLC–DAD procedure was proposed for determination of a multi-class mixture of nine drugs (AMX, MTZ, LVF, TNZ, DOX, FRZ, OMZ, PNZ and LNS) which are reported to be co-administered in different mixtures for the treatment of *Helicobacter pylori* infection. Nine compounds of different chemical structures and medicinal uses were effectively separated and accurately quantified in a run time less than 16 min; therefore, the described method can be considered cost- and time-effective. DAD was an advantageous and crucial element in the analysis. It served in dual functions: peak purity and homogeneity checker, in addition, the multiple wavelength detection enabled quantitation of each analyte at its optimum wavelength within the same run to boost sensitivity of measurement. The analytical method was successfully validated as per the ICH guidelines. The proposed method demonstrated various applications, first: the successful separation and estimation of the nine drugs in a single run, second: analysis of three different ternary mixtures of the drugs under investigation added to simulated intestinal fluid where good results were obtained with no matrix interference could be noticed and third: analysis of laboratory-prepared pharmaceutical formulations representing binary mixtures of the investigated drugs and acceptable recoveries were obtained. Finally, future perspectives for the proposed method include possible improvement and implementation in dissolution studies for single and multi-ingredient pharmaceutical products of the investigated drugs.

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** Declarations**

**Conflict of interest** Authors declare that they have no conflict of interest.

**Research involving human participants and/or animals** This article does not contain any studies with human participants or animals performed by any of the authors.

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