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Development of Novel HPLC Method for Analysing Drugs Used in H-Pylori Treatment

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ABSTRACT

Helicobacter pylori (H. pylori) is the most chronic bacterial infection on human being that is found in the gastric mucous layer and adapted to survive in acidic conditions. Because of the resistance of this organism, triple therapy treatment is required. In this study, RP-HPLC method was developed in terms of mobile phase composition, buffer concentration and additive amount to separate the complex drugs mixture used as triple therapy for H. pylori treatment. The first result showed that C18 reversed phase column has better resolution than C8 for analyzing amoxicillin, metronidazole, omeprazole and clarithromycin at isocratic elution mode. Design of experiments was then implemented to evaluate the best separation parameters. The results showed that amoxicillin was detected at 254 nm, where metronidazole, omeprazole and clarithromycin were detected at 304 nm. Moreover, better resolution was achieved at mobile phase compositions of 30:30:40 (acetonitrile: methanol: buffer), respectively. Regarding the optimum amount of trimethylamine added to the mobile phase to improve the resolution, the outcomes showed that 30 µL was the best choice at pH around 6.0 with 0.05 M potassium dihydrogen phosphate as a buffer. The developed method could separate the mixture as following; amoxicillin and omeprazole at 2.56, 4.84, respectively, where metronidazole and clarithromycin were retained 3.04 min.

Keywords: RP-HPLC, Design of Experiments, Triple therapy, H. pylori.

1. INTRODUCTION

H. pylori is the most chronic bacterial infection that is found in the gastric mucous layer and adapted to survive in acidic conditions inside the human body [1-3]. Because of the resistance of these bacteria, multidrug therapy is required [4]. H. pylori is responsible for gastric and duodenal ulcer disease that infects approximately 50% of the human population worldwide [5-6]. The predominant shape of this organism is curve or spiral [7-8] as shown in Figure.1. Peptic ulcers are localized erosion of the mucous membrane with a diameter of at least 0.5 cm of the stomach and duodenum [9]. The main causes of peptic ulcer disease are multifactorial such as Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or aspirin, alcohol and tobacco consumption [10], however, H. pylori is the one of the most important causes [11]. The pain related to ulcer is caused by irritation of exposed surfaces by the gastric acids [12].

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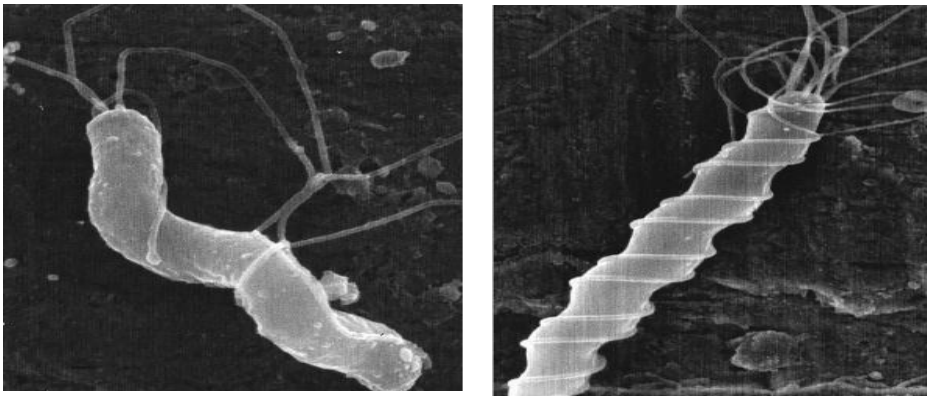

Figure.1. The predominant shape of H. pylori

The common medical treatment for ulcer caused by H. pylori requires a combination of at least three drugs (i.e. triple therapy). The recommended triple therapy consists of proton pump inhibitors (PPIs), mainly; omeprazole combined with two antibiotics such as: clarithromycin and amoxicillin or clarithromycin and metronidazole [12-13] with a time range of 10- 14 day [4]. Omeprazole (Figure 2-B) consists of 2-pyridyl methyl sulfinyl benzimidazoles with different substitutions on the pyridine or the benzimidazole [14-15]. It is considered as a weak base; hence, it can be used to regulate the acid production in the stomach [16-18]. Omeprazole inhibits the production of gastric acid by blocking the hydrogen-potassium adenosine triphosphate enzyme system (H^+, K^+ and the ATPase) (i.e. the proton pump which is essential for acid secretion by parietal cells that is the most acidic cell in the body) [19-20].

Metronidazole (Figure 2) belongs to a class of drugs called nitroimidazole that contains an imidazole ring [21]. In vivo, the NO_2 group on position 5 in metronidazole is reduced by bacteria and protozoan [22-23]. The reduction product is responsible for antimicrobial activity of metronidazole [22, 24-25]. Regarding clarithromycin (Figure 2), it belongs to a class of drug called macrolide that derived from erythromycin [26]. It is 6-O methyl erythromycin with a 14-membered macrocyclic lactone ring attached to two deoxy sugars (cladinose and desosamine) [27-28]. It is an acid stable antibiotic and well absorbed from the intestines where it shows an excellent antimicrobial activity that penetrates the stomach tissue [29-30]. Amoxicillin (Figure 2-D) is considered as an antibiotic derived from penicillin, 6-aminopenicillanic acid [31]. The chemical structures of the triple therapy drugs are shown in Figure 2, the chemical and physical properties are shown in Table 1.

Table 1. Chemical and physical properties of omeprazole, metronidazole, clarithromycin, and amoxicillin

Name of the drug	Omeprazole	Metronidazole	Clarithromycin	Amoxicillin
Chemical formula	$C_{17}H_{19}N_3O_3S$	$C_6H_9N_3O_3$	$C_{38}H_{69}NO_{13}$	$C_{16}H_{19}N_3O_5S$
Molar mass (g/mol)	345.42	171.15	747.95	419.64
Storage conditions	2-8 ºC	Room temperature	-20° C	2-8°C
Appearance	White powder	White powder	Off-White powder	White powder
Log P	2.23 [32]	-0.02 [33]	3.16 [35]	0.87 [36]

pKa	4.77 [32] 9.29	2.38 [34]	8.99 [35]	2.67 [36] 7.11 9.55
Solubility	Dissolve at alkaline pH. Soluble in methanol and has solubility in water of 0.52 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 5.92 mg/mL at 25°C.	Slightly soluble in methanol and has a solubility in water of 0.22 mg/mL at 25°C.	Slightly soluble in methanol and has solubility in water of 3.4 mg/mL at 25°C.
Name of the drug	Omeprazole	Metronidazole	Clarithromycin	Amoxicillin
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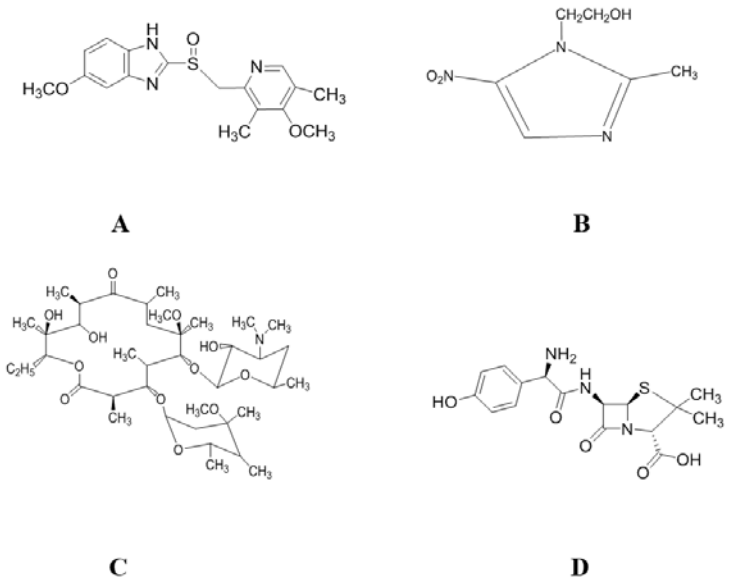

Figure 2. Chemical structures of: A) omeprazole, B) metronidazole, C) clarithromycin, and D) amoxicillin

High Performance Liquid Chromatography (HPLC) is an important tool for quantitative and qualitative analysis in pharmaceutical field [37-39]. Moreover, reversed phase high performance liquid chromatography (RP-HPLC) has been used in a wide range of separation in the field of pharmaceutical analysis, biology, proteomics and metabolomics [40-41], petroleum industry [41-42] and environmental analysis [41, 43], due to the wide range of commercial columns available.

Many studies reveal different methods for separating two or three of triple therapy drugs in a mixture using chromatographic techniques, where analytical methods have been developed for the quantitative analysis of these compounds, either alone or with combination with other drugs [31, 44-54]. For example, Walash et al., [49] developed new HPLC method for the determination of a ternary mixture of omeprazole, tinidazole and doxycycline in their raw materials and combined capsules using methanol and sodium dihydrogen phosphate at pH 4.5 using C18 column with retention time less than 6 min. El-khoudary et al., [51] used gradient elution in RP-HPLC to separate clarithromycin, norfloxacin, doxycycline, tinidazole and omeprazole in less than 9 min using ion pairing agent, phosphate buffer, and octanesulphonic acid with acetonitrile as a mobile phase. Moreover, Darwish et al., [48] also implemented C18 column to analyze tinidazole, doxycycline, omeprazole and clarithromycin within less than 10 min in a gradient elution mode using acetonitrile, methanol and potassium dihydrogen phosphate. Another study used C8 column for the separation of omeprazole, tinidazole and clarithromycin [52] in bulk powder and tablets using RP-HPLC in 5.44 min.

The present work aimed at developing a new methodology using HPLC-UV-Vis to analyze a mixture of four drugs used in H. pylori treatment at isocratic mode and studying the effect of different parameters; mobile phase composition, buffer concentration and amount of additive on the analysis with the help of Design of Experiments that had been implemented.

2. Experimental

2.1 Chemicals and reagents

All chemicals and solvents used were of analytical HPLC grade. Standards solutions of omeprazole, metronidazole, clarithromycin, and amoxicillin were purchased from Sigma-Aldrich, USA. Methanol was provided from Lichrosolv, Germany, acetonitrile was obtained from Sigma-Aldrich Co., France. Regarding trimethylamine, it was provided from TEDIA- USA, where LC water and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Sigma-Aldrich - Germany. Sodium hydroxide was supplied from Sigma Aldrich - Sweden, potassium hydroxide was provided from Riedel-de Haen and finally, ortho-phosphoric acid was provided from Scharlau- China.

2.2 Instrumentations

HPLC (Dionex, Germany) with Chromeleon software (Version 6.8) was used in this study. HPLC is equipped with autosampler injector system, quaternary pump, UV-VIS detector and column oven. The analysis was performed on two RP C18 and C8 columns: Thermo Scientific Hypersil BDS C18 of (250 × 4.6 mm, 5µm) and Hypersil BDS C8 (150 × 4.6 mm, 5µm). The experimental design was carried out using Minitab, version 18. UV-Visible spectrometer (double beam- Cary E100) was used to get the maximum wavelength for the compounds.

2.3 Preparation of standard solutions

Stock solutions of omeprazole, metronidazole, clarithromycin and amoxicillin at 1000 ppm were prepared separately by dissolving 0.1g of each compound in: 100 mL methanol for metronidazole and clarithromycin, 100 mL water for amoxicillin, where omeprazole was dissolved in few drops 0.1 M NaOH then completed to 100 mL with methanol. The solution was then sonicated for 5 min. Regarding the standard mixture of the four drugs; the 100-ppm solution was prepared in pure water. Buffer solution of KH_2PO_4 was prepared in 500 mL at two

concentrations: 0.02 and 0.05 in water.

2.4 Design of Experiments (DOE)

Different HPLC conditions were evaluated on C18 column: composition of the mobile phase, buffer concentration and amount of additive added to the mobile phase. HPLC analysis was performed using isocratic elution with constant flow rate and injection volume during the whole experiments at 1.0 mL/min and 10 µL, respectively. Minitab software was used to create the DOE.

3. Results and Discussion

3.1 Detection of the wavelength maxima

The UV-Vis absorption spectrum of drugs was found in the region between 200– 800 nm as shown in Figure 3. The 205 nm has not been selected in this work since it showed interference with the mobile phases used in this study. Therefore, the suitable wavelength for the studied drugs was performed at 254 nm for amoxicillin and 304 nm for metronidazole, clarithromycin and omeprazole.

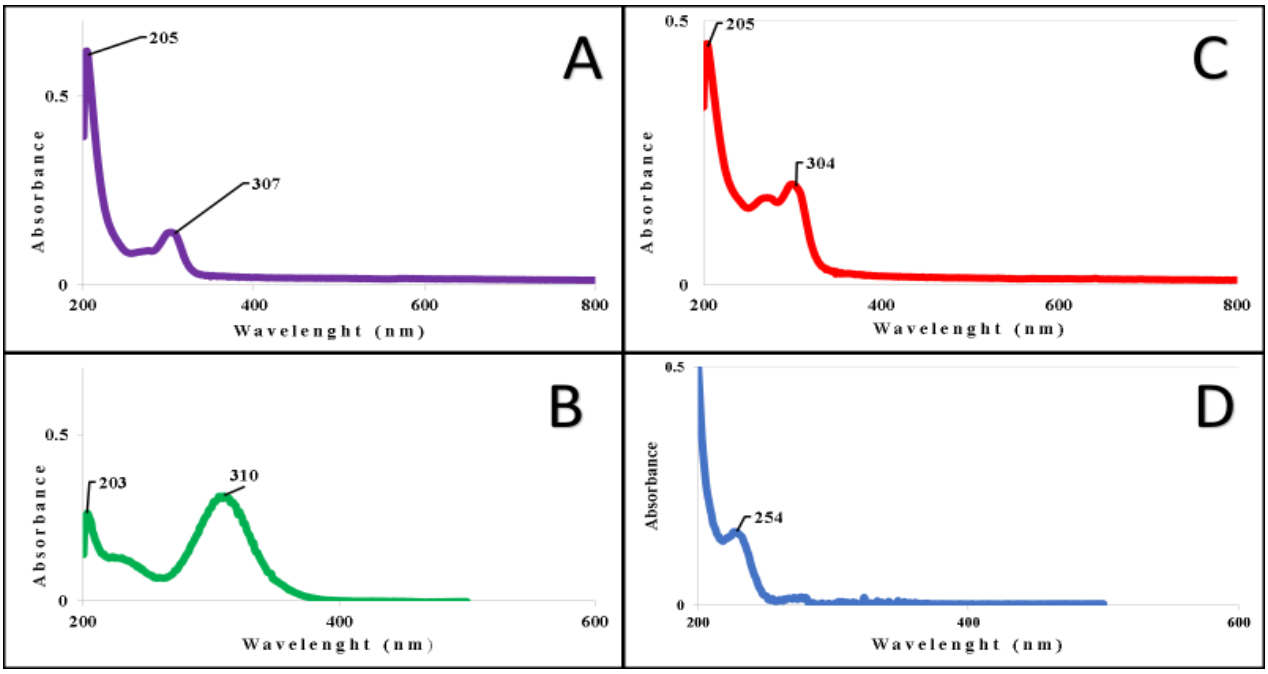

Figure 3. UV-Vis absorption spectra of (A) clarithromycin, (B) metronidazole, (C) omeprazole and (D) amoxicillin.

3.2 Selection of HPLC Column

As a preliminary test to choose the best stationary phase, a mixture of omeprazole, clarithromycin, amoxicillin and metronidazole was injected on HPLC. The results are presented in Figure 4.

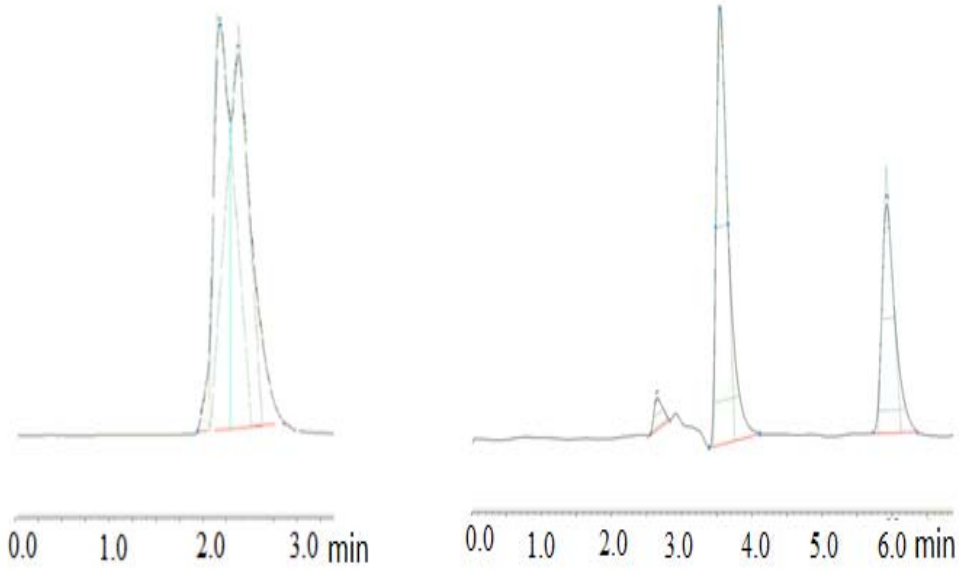

Figure 4. HPLC chromatograms of amoxicillin, clarithromycin, metronidazole and omeprazole at (33:17:50) ACN: MeOH: KH_2PO_4, respectively at 254 nm, flow rate 1.0 mL/min, injection volume 10 µL at room temperature. The tested columns are: A) C8 Hypersil BDS (150 x 4.6 mm. 5µ), and B) C18 Thermo scientific hypersil BDS (250 x 4.6 mm. 5µ).

The preliminary results showed that the C8 detected two peaks with retention time of less than 3.0 min, which was insufficient for the solutes to interact with the stationary phase and to separate them in proper resolutions. On the other hand, C18 column could detect three peaks with a retention time of around 6 min.

Hence, based on the preliminary test, Thermo Scientific Hypersil BDS C18 column was selected to continue the experimental work.

3.3 Effect of temperature

As known, increasing the temperature in HPLC analysis will decrease the viscosity, and then the retention time. Hence, in order to study the effect of mobile phase composition, concentration of buffer and the amount of additive on the analysis, the temperature should be determined and fixed. Herein, two temperatures: room temperature and 30°C have been tested on the chosen Thermo Scientific Hypersil BDS C18 column. The preliminary results showed that changing the temperature from room temperature to 30°C has very small effect on the separation asymmetric factor (As) and the plate height (H) (Table 2), it affects only the retention time as shown Figure 5 (A, B).

Therefore, we decided to implement the room temperature in the next steps.

Table 2. Separation results of the drugs mixture of amoxicillin and clarithromycin at different temperatures.

Condition	Amoxicillin			Clarithromycin		
	tr (min)	As	H	tr (min)	As	H
MeOH:KH_2PO_4 (25:75, v/v) 30°C	4.200	0.83	2.18	7.20	4.47	22.88
MeOH:KH_2PO_4 (25:75, v/v) at room temperature	4.600	0.86	2.03	7.50	3.96	22.77

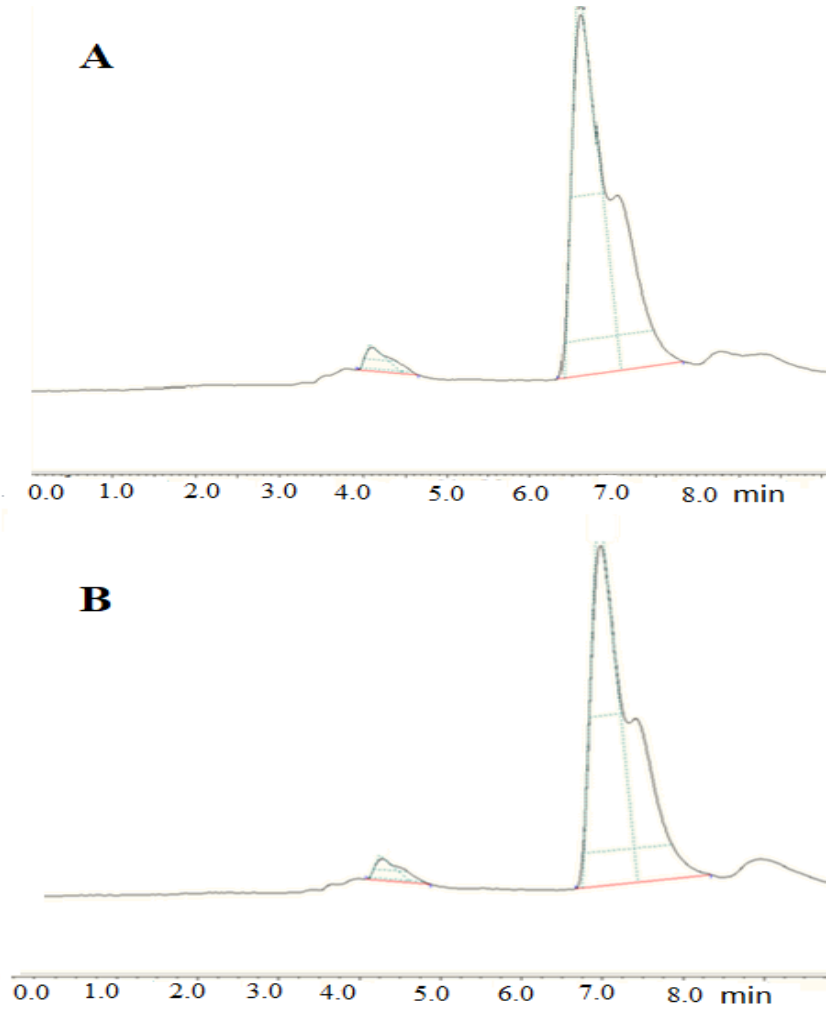

Figure 5. Chromatograms of amoxicillin and clarithromycin at pH 5.8, MeOH: buffer (25:75, v/v), flow rate 1.0 mL/min, injection volume 10 µL using C18 (250 × 4.6 mm, 5µm) column.

A) at 30°C and B) at room temperature.

3.4 Design of Experiments (DOE)

After choosing C18 Thermo Scientific Hypersil BDS column and room temperature as running parameters, Design of Experiments was built accordingly. The studied factors were: mobile phase composition, triethylamine

(TEA) amount and buffer concentration in order to choose the best parameters. For each condition, replicate samples have been injected and the average of results was registered. The results for these conditions were summarized in Table 3.

Table 3. DOE for the separation of amoxicillin, clarithromycin, metronidazole and omeprazole using C18 (250 x 4.6 mm, 5µ) column, flow rate of 1.0 mL/min at room temperature.

(ACN: MeOH: buffer) %	TEA (µL)	[buffer]	tr (min)
30:20:50	0	0.02	5.121 [A]* 6.408 [B & C]* 14.463 [D]*
30:20:50	0	0.05	2.559 [A] 3.211 [B & C] 7.019 [D]
30:20:50	30	0.05	5.097 [A] 6.361 [B & C] 13.656 [D]
30:20:50	30	0.02	2.553 [A] 3.165 [B & C] 6.334 [D]
20:20:60	0	0.02	2.649 [A] 3.604 [B & C] 15.842 [D]
20:20:60	0	0.05	2.658 [A] 3.706 [B & C] 18.992 [D]
20:20:60	30	0.02	2.419 [A] 2.662 [B & C] 17.478 [D]
20:20:60	30	0.05	2.668 [A] 3.628 [B & C] 15.772 [D]
20:30:50	30	0.02	3.417 [A] 4.473 [B, C & d]
20:30:50	30	0.05	2.597 [A] 3.244 [B & C] 7.875 [D]
20:30:50	0	0.05	2.724 [A] 3.358 [B & C] 9.657 [D]

(ACN: MeOH: buffer) %	TEA (µL)	[buffer]	tr (min)
20:30:50	0	0.02	2.638 [A] 3.322 [B & C] 9.564 [D]
30:30:40	0	0.02	4.966 [A] 6.068 [B & C] 9.955 [D]
30:30:40	0	0.05	2.653 [A] 3.054 [B & C] 5.275 [D]
30:30:40	30	0.02	2.502 [A] 3.039 [B & C] 5.122 [D]
30:30:40	30	0.05	2.562 [A] 3.036 [B & C] 4.839 [D]

*A: Amoxicillin, *B: clarithromycin, *C: metronidazole and *D: omeprazole.

Based on the experiments of the DOE, the best chromatogram observed was at (30: 30: 40) % ACN: MeOH: buffer, 30µL TEA, buffer concentration of 0.05 M (fixed at pH 6), since these conditions showed the best results in terms of reasonable retention time, less degradants peaks for amoxicillin and better peak shape compared to the other conditions (Figure 6).

Adding triethylamine (TEA; as ion pair reagent) to the mobile phase prevents tailing and band broadening of the peaks [48, 53]. In our results, when 30 µL TEA was added in comparison to 0 µL, the peak shape is enhanced, this is refers to the fact that TEA interacts strongly with silanols and inhibits them from interacting with amines in the drugs sample.

Regarding the buffer concentration, 0.05 M showed the best choice to improve the peak shape in comparison to 0.02M due to the increase of the ionic strength. Our result was in agreement with Darwish et al., [48] who studied the effect of buffer concentration (0.01, 0.03, 0.05 and 0.06 M). They found that 0.03 M was the best choice in comparison to 0.01 M since the peak shape enhanced due to ionic strength.

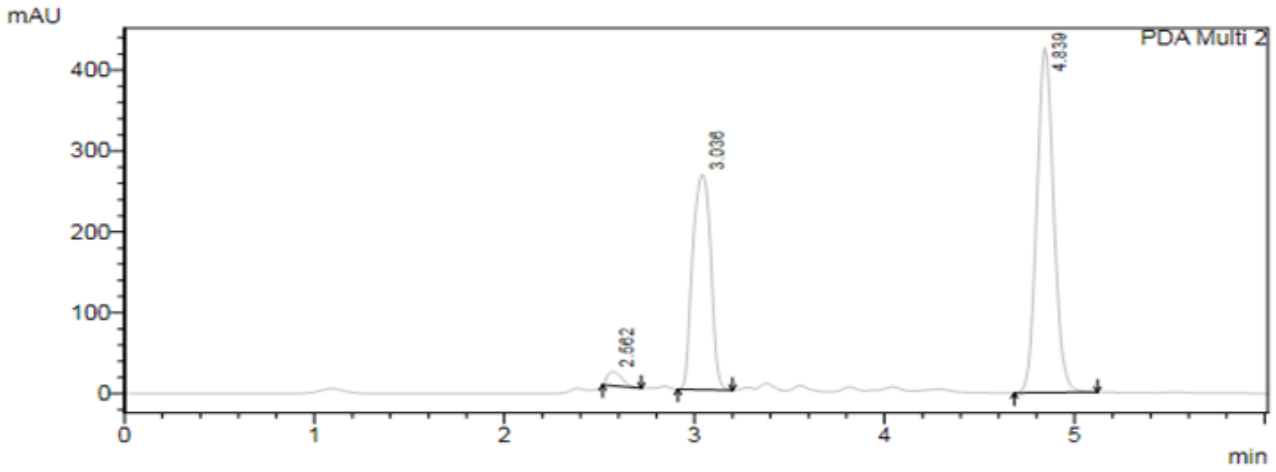

Figure 6. Chromatogram of amoxicillin (2.562 min), clarithromycin and metronidazole (3.036 min) and omeprazole (4.839 min) at (30: 30: 40)% of ACN: MeOH: buffer, respectively. A 30µL TEA at pH 6.0, flow rate of1.0 mL/min, injection volume 10 µL at room temperature using C18 (250 x 4.6 mm, 5µ) column at isocratic mode.

Conclusion

RP-HPLC method was developed in terms of mobile phase composition, buffer concentration and additive amount to separate the drugs mixture used as triple therapy for H. pylori treatment. A C18 reversed phase column showed better resolution than C8 for analyzing amoxicillin, metronidazole, omeprazole and clarithromycin at isocratic elution mode. The mobile phase compositions of 30:30:40 (ACN: MeOH: buffer) %, respectively showed the best choice in comparison to the other conditions. Regarding the optimum amount of trimethylamine added to the mobile phase to improve the separation, the outcomes showed that 30 µL was the best choice at pH around 6.0 with 0.05 M potassium dihydrogen phosphate as a buffer. The developed method could separate the mixture as following; amoxicillin and omeprazole at 2.56, 4.84, respectively, where metronidazole and clarithromycin were retained 3.04 min. Further investigations will be carried out in the future to separate metronidazole and clarithromycin.

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Conflict of interests

The authors declare that they have no conflict of interest

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تطوير طريقة جديدة باستخدام استِشراب السَّائِل الرَّفيع HPLC لتحليل الأدوية المستخدمة في علاج الجرثومة الملوية البوابية

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ملخص

الجرثومة الملوية البوابية (H-pylori) هي أكثر أنواع العدوى البكتيرية المزمنة التي تصيب الإنسان وتوجد في الطبقة المخاطية في المعدة وتتكيف مع الظروف الحمضية. بسبب مقاومة هذه الجرثومة، فإن العلاج الثلاثي مطلوب. في هذه الدراسة ، تم تطوير طريقة فصل باستخدام طريقة استشراب السائل الرفيع – الطور الثابت الغير قطبي، من حيث تكوين الطور المتحرك وتركيز مادة معادلة الحموضة والكمية المضافة لفصل خليط الأدوية المعقد المستخدم كعلاج ثلاثي لعلاج بكتيريا الملوية البوابية. أظهرت النتيجة الأولى أن عمود الطور الثابت الغير قطبي C18 له فاعلية فصل أفضل من C8 لتحليل : أموكسيسيلين وميترونيدازول وأوميبرازول وكلاريثروميسين. ثم تم تنفيذ تصميم التجارب لتقييم وتحديد أفضل ظروف الفصل. أظهرت النتائج أن الأموكسيسيلين تم فصله عند 254 نانومتر ، بينما تم الكشف عن ميترونيدازول وأوميبرازول وكلاريثروميسين عند 304 نانومتر. علاوة على ذلك ، تم تحقيق اعلى فاعلية فصل في تركيبات الطور المتحرك عند استخدام النسبة 30:30:40 (أسيتونيتريل: ميثانول: منظم الحموضة) ، على التوالي. فيما يتعلق بالكمية المثلى من ثلاثي ميثيل أمين المضاف إلى الطور المتحرك لتحسين فاعلية الفصل ، أظهرت النتائج أن 30 ميكرولتر كان الخيار الأفضل عند درجة الحموضة 6.0 مع 0.05 مول/لتر من فوسفات ثنائي هيدروجين البوتاسيوم كمنظم للحموضة. يمكن للطريقة المطورة فصل الخليط على النحو التالي؛ أموكسيسيلين وأوميبرازول عند 2.56 ، 4.84 دقيقة، على التوالي، بينما تم فصل الميترونيدازول والكلاريثروميسين عند الزمن 3.04 دقيقة.

الكلمات الدالة: استشراب السائل الرفيع بالطور الغير قطبي ، تصميم التجارب ، العلاج الثلاثي ، الحلزونية البوابية.

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