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ESTIMAÇÃO SIMULTÂNEA DE CLONAZEPAM E METRONIDAZOL EM COMPRIMIDOS FARMACÊUTICOS PELO MODO DE CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA DE FASE REVERSA COM DETECÇÃO UV

SIMULTANEOUS ESTIMATION OF CLONAZEPAM AND METRONIDAZOLE IN PHARMACEUTICAL TABLETS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY MODE WITH UV DETECTION

التقدير التلقائي لكلونازيبام ومترونيدازول في الأقراص الصيدلانية بطريقة كروماتوغرافيا السائل عالي الأداء باستخدام مكشاف الأشعة فوق البنفسجية

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RESUMO

O clonazepam (CLO) desempenha um papel significativo no tratamento de convulsões, convulsões mioclônicas e outras infecções clínicas, enquanto o metronidazol (MTZ) é um medicamento antiprotozoário e antibacteriano. A técnica de cromatografia líquida de alta eficiência em fase reversa (RP-HPLC) tem proporcionado maior precisão e sensibilidade em relação a outros métodos, principalmente o colorimétrico e espectrofotométrico. O objetivo deste estudo foi implementar um método simples em comprimidos puros e farmacêuticos para a determinação simultânea de CLO e MTZ. O desenvolvimento e otimização do método RP-HPLC para validar um método de separação para a estimativa simultânea de ambos os fármacos na formulação farmacêutica envolveu o estudo da fase móvel ideal, concentração do tampão e valor de pH. Sob as condições cromatográficas, o sistema RP-HPLC obteve excelente separação em um Phenomenex HyperClone BDS (250 x 4,60 mm, 130A e 5µ) a uma temperatura de 45 °C e nas seguintes condições: 65: 35% de acetonitrila: ácido acético e solução tampão de acetato de sódio (NaOAc / HAc) como fase móvel (pH 3,5); volume de injeção de 20 µL a uma taxa de fluxo de 0,75 mL / min e comprimento de onda de detecção de 310 nm. O procedimento de RP-HPLC proposto demonstrou alta precisão (RSD% <1%), bem como a boa relação linear do gráfico de calibração nas faixas de concentração de 50-160, 35-100 ppb com um coeficiente de determinação (r^2) do linha de regressão de 0,9996 e 0,9997 para metronidazol e clonazepam, respectivamente. O método proposto ofereceu excelentes valores validados tanto para LOD (4,24 e 3,06 ppb) e LOQ (14,15–10,21 ppb) para metronidazol e clonazepam, respectivamente. O método RP-HPLC foi aplicado com sucesso para estimar metronidazol e clonazepam nos conhecidos comprimidos farmacêuticos comerciais e deu uma recuperação excelente (> 98%) para ambos os medicamentos. Os resultados do método foram comparados com o método farmacopéico padrão para ambas as drogas por meio de testes estatísticos, que não indicaram diferença na precisão entre os métodos

Palavras-chave: *Clonazepam, metronidazol, fase reversa, HPLC, preparações farmacêuticas.*

ABSTRACT

Clonazepam (CLO) plays a significant role in treating seizures, myoclonic seizures, and other clinical infections, while metronidazole (MTZ) is an antiprotozoal, antibacterial drug. The reversed-phase high performance liquid chromatographic (RP-HPLC) technique has provided greater precision and sensitivity over other methods, especially the colorimetric and spectrophotometric. This study aimed to implement a simple method in pure and pharmaceutical tablets for the simultaneous determination of CLO and MTZ. The RP-HPLC method's development and optimization for validating a separation method for the simultaneous estimation of both drugs in pharmaceutical formulation involved studying the optimum mobile phase, buffer concentration, and the pH value. Under the chromatographic conditions, RP-HPLC system achieved excellent separation on a Phenomenex HyperClone BDS (250 x 4.60 mm, 130A, and 5µ) at a temperature of 45°C and the following conditions: 65:35% acetonitrile:acetic acid and sodium acetate buffer solution (NaOAc/HAc) as

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mobile phase (pH 3.5); 20 µL injection volume at a flow rate of 0.75 mL/min and detection wavelength of 310 nm. The proposed RP-HPLC procedure demonstrated high precision (RSD% < 1%), as well as the good linear relationship of the calibration graph at concentration ranges of 50-160, 35-100 ppb with a coefficient of determination (r^2) of the regression line of 0.9996 and 0.9997 for metronidazole and clonazepam respectively. The proposed method offered excellent validated values for both LOD (4.24 and 3.06 ppb) and LOQ (14.15–10.21 ppb) for both metronidazole and clonazepam drugs, respectively. The RP-HPLC method has been successfully applied to estimate metronidazole and clonazepam in the well-known commercial pharmaceutical tablets and gave an excellent recovery> 98% for both drugs. The results of the method was compared with the standard pharmacopeial method for both drugs using statistical tests, which indicated no difference in accuracy between the methods.

Keywords: *Clonazepam, metronidazole, reversed-phase, HPLC, pharmaceutical preparations*

الملخص:

ان التأثير الفعال للكلونازيبام هوفي علاج النوبات المصحوبة بغيبوبة ونوبات الرمع العضلي وغيرها من المؤشرات السريرية. في حين أن الميترونيدازول هو دواء مضاد للجراثيم. قدمت تقنية الكروماتوغرافيا السائلة ذات الطور العكسي (RP-HPLC) قدرًا أكبر من التوافقية والحساسية على الطرق الاخرى وخاصة طرق القياس اللوني والطيف الضوئي. تشرح المقالة كيفية تطوير طريقة RP-HPLC الحساسة التي يمكن تطبيقها للتقدير التلقائي للميترونيدازول والكلونازيبام في المستحضرات الصيدلانية. تم دراسة الظروف المثلى لطريقة RP-HPLC للتحقق من صحة طريقة الفصل لتقدير لكلا العقارين في المستحضر الصيدلاني ، ودراسة الطور المتحرك الامثل وتركيزالبفر ودرجة الحموضة. في ظل الظروف الكروماتوغرافية ، حقق نظام RP-HPLC فصلًا ممتازًا على Phenomenex HyperClone BDS (250 × 4.60mm,) بابعاد 130 انكستروم ، و 5 مايكرون) عند درجة حرارة 45 درجة مئوية ، باستخدام (35:65) ٪ أسيتونيتريل: محلول بفر(حمض الأسيتيك وأسيتات الصوديوم (NaOAc / HAc)) كطور متحرك (درجة الحموضة 3.5) ، حجم حقن 20 ميكرولتر بمعدل سريان 0.75 مل / دقيقة ، بينما كان الطول الموجي للكشف 310 نانومتر. أظهرت طريقة RP-HPLC المقترحة دقة عالية (RSD٪ <1٪) ، بالإضافة إلى العلاقة الخطية الجيدة لمنحني المعايرة عند التركيز من(160-50) و (100-35) جزء في البليون مع معامل ارتباط (**r2**) ذو قيمة 0.9996 و 0.9997 للميترونيدازول والكلونازيبام على التوالي. قدمت الطريقة المقترحة قيمًا ممتازة لحد الكشف مقداره4.24 و 3.06 جزء في البليون) وحد الكمية فكانت القيم (14.15 و10.21 جزء في البليون) لكل من أدوية الميترونيدازول وكلونازيبام على التوالي. تم تطبيق طريقة RP-HPLC بنجاح لتقدير الميترونيدازول والكلونازيبام في الأقراص الصيدلانية التجارية المعروفة وأعطت قيم استرداد ممتازة > 98٪ لكلا العقارين على التوالي. تم مقارنة نتائج الطريقتين مع طرق دستور الادوية القياسية بواسطة الاختبارات الاحصائية التي بينت عدم وجود فرق بالدقة بين هذه الطرق.

الكلمات المفتاحية: الكلونازيبام، المترونيدازول, الطور العكوس, كروماتوغرافيا السائل العالي الاداء, المستحضرات الصيدلانية

1. INTRODUCTION:

Clonazepam (CLO) (Figure 1) chemically known as 5-(2-chlorophenyl)-7-nitro-1H-enzo [e] [1,4]diazepin-2(3H)-one. CLO is a benzodiazepine medication used to relax the brain and nerves with anticonvulsants for many epilepsy, anxiety, and schizophrenia (Hart, Gourley, and Herfindal, 1992; Sweetman, 2009). CLO activity contributes to enhanced GAMMA receptor responses to aminobutyric acid (Lehoullier and Ticku, 1987; Riss, Cloyd, Gates, and Collins, 2008; Seubert and Saad Rasheed, 2017; Skerritt and Johnston, 1983).

The CLO half-life in plasma has varied from 19 to 60 h, where the mean value is 40 hr (Steentoft and Linnet, 2009). CLO has a variety of clinical indications, including sedative, anti-anxiety, anticonvulsant, and muscle relaxant. However, it is one of the Benzodiazepine with high potency and potentially addictive.

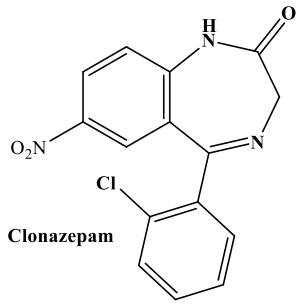

Figure 1. *Structures formal of CLO*

Therefore, the delicate balance between using and misusing is always a concern in medical uses (Al-Phalahy, Muhamad, and Rasheed, 2016; Longo and Johnson, 2000). CLO oral tablet may cause drowsiness, addictive, and it can cause other side effects as well. This medication will slow the movement, thought, and reaction time of the brain. Clonazepam oral tablet may have a more severe side effect: drowsiness, walking and balance difficulties, dizziness, insomnia, exhaustion, and memory issues (Lee-Chiong, 2008; Sorel, Mechler, and Harmant, 1981; Wollman, Lavie,

and Peled, 1985).

Due to the therapeutic significance of CLO, several techniques for determining it in pharmaceutical and/or biological forms have been developed. HPLC technique is extensively used for the assay of drugs and other compounds. The literature involved several HPLC methods for estimation of CLO, and most of these methods use the C18 column (Bares, Pehourcq, and Jarry, 2004; El Mahjoub and Staub, 2000; Ibrahim, El-Enany, Shalan, and Elsharawy, 2016; Meghana, Lahari, Kumari, and Prakash, 2012; Patil, Wankhede, and Chaudhari, 2015), with different mobile phase such as acetonitrile:0.01M sodium acetate (Bares *et al*., 2004), acetonitrile: phosphate buffer (El Mahjoub and Staub, 2000; Meghana *et al*., 2012), acetonitrile: methanol (60:40 v/v) (Patil *et al*., 2015), and sodium dodecyl sulfate: 12% n-propanol in phosphoric acid (Ibrahim *et al*., 2016).

Reversed-phase high-performance liquid chromatography (RP-HPLC) (reversed to normal HPLC) involved non-polar or hydrophobic stationary and polar mobile phases. As a result, the high polarity compounds are eluted earlier than non-polarity or low polarity, which retained for a longer time. The columns most widely used for reversed-HPLC separations are octyl (C_8), octadecyl (C_{18}), phenyl, Octadecyl silane (ODS), or cyanopropyl chemically bonded to microporous silica particles (Hodges and Mant, 1991; Mant, Cepeniene, and Hodges, 2010). On the other hand, various mixtures of solvents are used as mobile phases such as acetonitrile, water, and methanol), thus playing an essential role in the efficient separation process (Henry, 2009; Unger and Liapis, 2012). RP-HPLC provides many advantages, such as high speed (analysis process can be done in a few minutes), good sensitivity (various detectors can be used), and reusable columns (Dong, 2013).

Metronidazole (MTZ) (Figure 2) is chemically known as 2-(2-methyl-5-nitro-1H-imidazole-1-yl) ethanol (Naveed, Waheed, and Nazeer, 2014) and extensively used in clinical treatment. The discovery of the antitrichomonal properties of antibiotic azomycin led to the investigation of nitroimidazoles as antiparasitic agents (Edwards, 1993; Pharmacopoeia, 2016). MTZ is a medicine for amebiasis (liver and colon), giardiasis (small bowel), and trichomoniasis that can be used as a treatment option (Katzung and Trevor, 2015; Reynolds and Parfitt, 1993).

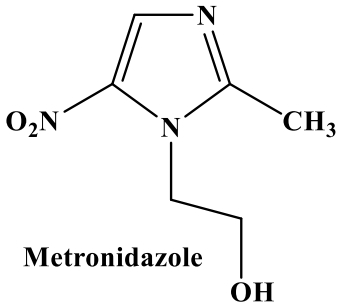

Figure 2. *Structures formal of MTZ*

MTZ was used against anaerobic organisms, anaerobic bacteria, amoebozoa infections, and antiprotozoal (Peyghan, Powell, and Zadkarami, 2008; Wilson, Gisvold, Block, and Beale, 2004). The initial clinical tests of MTZ indicated that it was capable of curing Helicobacter pylori (peptic ulcer diseases) and treating invasive amoebic dysentery (Rossi, 2013). MTZ antimicrobial activity is due to the presence of a nitrogen group that has been decreased chemically by bacterial inhibition due to anaerobic bacteria and protozoans and resulting in bacterial cell death (Chrystal, Koch, McLAFFERTY, and Goldman, 1980). The resulting product is responsible for the MTZ antimicrobial activity and the parasite DNA interaction of the MTZ (Eisenstein and Schaechter, 2007). The toxicity of the central nervous system can be pancreatitis and serious. MTZ is a little well tolerated but can produce a spectrum from peripheral neuropathy, cerebellopathy, encephalopathy, and epilepsy to adverse neurological effects. The use of alcohol for MTZ therapy can lead to nausea and vomiting (Hwang *et al*., 2012; Kim *et al*., 2004; Kuriyama, Jackson, Doi, and Kamiya, 2011).

Several HPLC methods for evaluating MTZ in pharmaceutical forms have been developed. These methods involved use Hypersil BDS C18, 150x4.6, 5µ as stationary phase with phosphate buffer and acetonitrile : (90:10 %v/v) as a mobile phase, while the other method used C_{18} column (250x 4.6 mm, 5µm) with Acetonitrile: 0.5 M potassium dihydrogen orthophosphate buffer pH 4.5 with triethylamine 30:70 (v/v) as mobile phase, with a wavelength of 289 nm (Al-Abachi, Abed, and Alamri, 2020; Ghante, Pannu, Loni, and Shivsharan, 2012). No analysis of the retention characteristics of CLO and MTZ on a Phenomenex HyperClone BDS (250 x 4.60 mm, 130A, and 5µ), has been conducted, given the availability of numerous works of separation CLO and MTZ on HPLC.

This study aimed to implement a simple method in pure and pharmaceutical tablets for the simultaneous determination of CLO and MTZ.

2. MATERIALS AND METHODS:

2.1. Chemicals

Metronidazole and clonazepam and as standards were donations from SDI, Samara, Iraq. Acetonitrile (ACN), sodium acetate, acetic acid were HPLC grade and were obtained from Sigma-Aldrich. For applications, pharmaceutical tablets were obtained from a pharmacy to different companies (Julphar-UAE, SDI-Iraq, Hoffman-LaRoche-Switzerland, and Roche Farma-Spain).

2.2. HPLC apparatus

The chromatographic system Merck-Hitachi was equipped with a flow pump Model L-6200, a Model L-4200 UV/Vis detector, and an N2000 Photographic Data Workstation Module Integrator. The chromatographic separation was performed with an RP-C8 stationary phase Phenomenex HyperClone BDS 250 x 4.60 mm, 130A, and 5µ). The mobile phase, acetonitrile–10 mM sodium acetate buffer (pH = 3.5)–(35: 65, v/v) was controlled at a flow rate of 0.75 mL/min at 45 °C. A 310 nm UV detector tracked the column effluent.

2.3. Preparation of standard solutions

A stock solution of CLO or MTZ (1000 µg mL^{-1}) was prepared by dissolving 0.100 g in 100 mL of acetonitrile. More working standard solutions were prepared by dilution of the stock solution with the same solvent to the desired concentration. From the stock solutions of clonazepam and metronidazole, a working solution (200 ppb) was made-up in millipore water in a brown flask. The calibration standards were prepared freshly from this working solution. The calibration standards of clonazepam used were 35, 52, 68, 85, and 100 ppb whereas for the metronidazole the final concentrations were 50, 78, 105, 130, and 160 ppb.

2.3.1 Preparation of CLO and MTZ tables

A total of twenty-five of CLO tablets containing 2 mg of commercial drugs were weighed. The average weight was calculated and powdered. After shaking well and pouring into a 50 ml volumetric flask, an equal quantity of 50 mg CLO was dissolved in 30 mL of ACN for waste. The residue has been ACN washed, and the amount with ACN has gradually been raised to 50 ml. Twenty-five MTZ tablets containing 500 mg of the commercial drug were weighed, and the average weight was calculated. Then, the tablets were powdered. After well-filtering and residue disposal, the quantity equivalent to 100 mg MTZ was dissolved in 10 mL. The residue was washed with ACN, and the amount with ACN eventually reached 20 mL. Subsequently, the solution was filtered by filters (0.45 µm).

2.5. Method development

The sensitive and straightforward RP-HPLC method using C_8 stationary phase Phenomenex HyperClone BDS (250 x 4.60 mm, 130A, and 5µ) and 0.75 mL/min as a flow rate in addition to acetic acid and sodium acetate buffer solution as mobile phase-ACN(NaOAc/HAc) was developed and validated for the simultaneous determination MTZ and CLO. To achieve an effective RP-HPLC method with high resolution and efficient separation, significant factors were optimized. In addition to the MTZ and CLO retention behavior, various acetonitrile containing, buffer levels, and pH eluent have been examined. The detection was carried at 310 nm. And the conditions were adjusted for the construction of calibration curves to estimate both drugs MTZ and CLO.

3. RESULTS AND DISCUSSION:

3.1. Separation of clonazepam and metronidazole

In RP-HPLC system development and quantitative estimation, the separation process is a preliminary stage. The main objective of this project was to select a clear approach that ensures that MTZ and CLO are goodly separated. A mobile NaOAc / HAc buffer phase with a varying ACN content on the RP column was chosen as test pharmaceuticals by the CLO and MTZ for a study on their retention mechanism in RP-mode. Figure 3 displays the chromatogram. The chromatogram was obtained in a NaOAc / HAc buffer with 35% ACN and 10 mM (pH 3.5).

3.2. The effect of ACN percent on retention of MTZ and CLO

Mobile phase compositions are changed systemically by variation of the ACN content from 35% to 95% (v/v) with constant the concentration of the buffer 10 mM at pH 3.5 (Figure 4). The

pharmaceutical CLO shows behavior hydrophobic interaction with increasing ACN content in the mobile phase. Otherwise, MTZ shows behavior hydrophilic (HILIC). The hydrophilicity of the drug compounds is responsible for this difference in behavior. The values of the pharmaceuticals are evident from the $logP_{ow}$. This is explained in $logP_{ow}$ MTZ and CLO values, respectively (-0.46, 3.15) (Sangster, 2010).

3.3. The effect of buffer pH on retention of MTZ and CLO

The buffer pH was varied from 3.5-5.5 with constant the concentration of the buffer 10 mM at ACN content 35% (Figure 5). The retention factor of CLO and MTZ decreased with increasing pH buffer from 3.5 to 5.5. The source of this behavioral difference is the isoelectric point (9.22, 6.77) values of MTZ and CLO, respectively.

3.4. The effect of buffer concentration on retention of MTZ and CLO

At the end of optimization conditions, the buffer concentration was changed from 10 to 30 mM with constant the ACN content 35% at pH 3.5, and therefore, we found no significantly altered (Figure 6).

3.5. Calibration graph

MTZ and CLO calibration graphs have been developed in optimal conditions (eluent: (10 mM sodium acetate, pH 3.5, 35% acetonitrile), 310 nm UV detection, 20 µL injection volume, flow rate 0.75 mL/min, 45°C temperature) by plotting area versus to CLO and MTZ concentrations and display the 50 -160 and 35-100 ppb range concentration of MTZ and CLO, respectively (Figure 7).

3.6. Statistical data analysis

The direct calibration graphs were constructed, and the statistical results are shown under Table 1 for the direct determination of CLO and MTZ in RP-Mode. The approach has been ICH-validated (Guideline, 2005) for two concentrations covering the range. Each concentration was repeated (n=3) and five consecutive days of calibration samples analyzed. The accuracy and the precision of RSD and recovery measurements were measured in one run (intra-day) and in between (inter-day) assays (Table 2).

3.7. Determination of CLO and MTZ in pharmaceutical tablets

Two of the pharmaceutical preparations containing the target drugs (tablet) with the stated concentration of 2 and 500 mg respectively per unit are successfully used in MTZ and CLO determinations, and the obtained results are described in Table 3. The data obtained using the RP-mode (Table 3) was compared with the standard method (Pharmacopeia, 2009) using the 95% confidence student t-test test and variance F-test. The t- and F-values (Tables 4) calculated did not exceed the theoretical values which indicate that the accuracy and precision method for determining MTZ and CLO in pharmaceutical formulations did not differ significantly.

4. CONCLUSIONS:

The proposed method was validated for precision, accuracy, specificity, reproducibility, and robustness for simultaneous quantitative estimation of MTZ and CLO in dosage forms. The chromatographic conditions were investigated to achieve good separation efficiency. Retention times of approximately 3 and 6 minutes for MTZ and CLO, respectively, were recorded on the C_8 stationary phase Phenomenex HyperClone BDS (250 x 4.60 mm and 5µ) column. The proposed RP-HPLC method was applied for the determination of the mentioned drugs (MTZ and CLO) in pharmaceutical formulations with high accuracy and precision. Therefore the proposed RP-HPLC method can be used for routine extermination of MTZ and CLO in pharmaceutical dosages.

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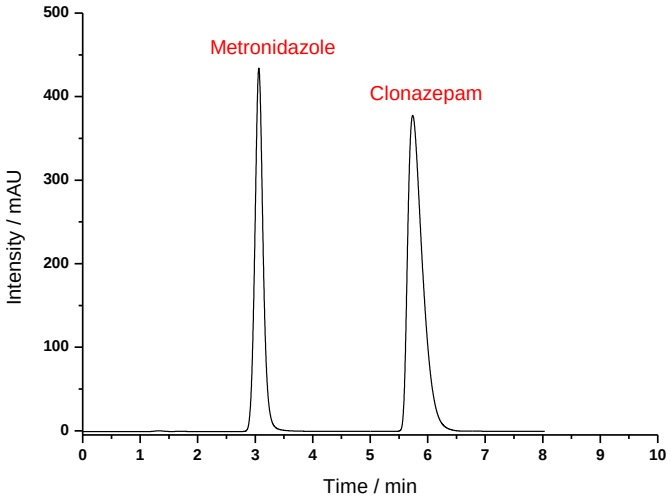

Figure 3. *Chromatogram for MTZ and CLO*

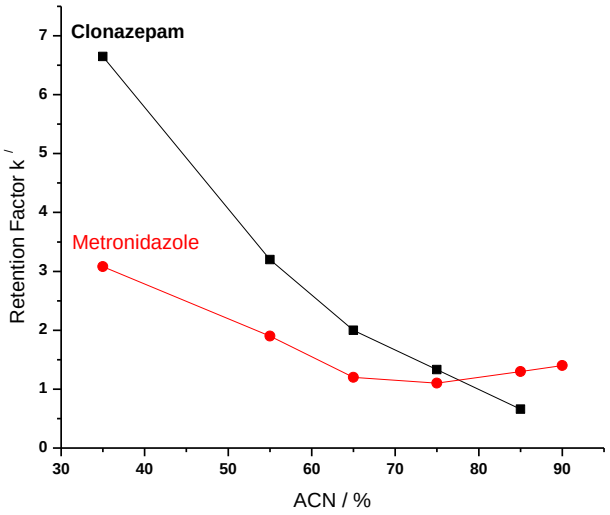

Figure 4. *Effect of ACN ratio on retention factor for MTZ and CLO.*

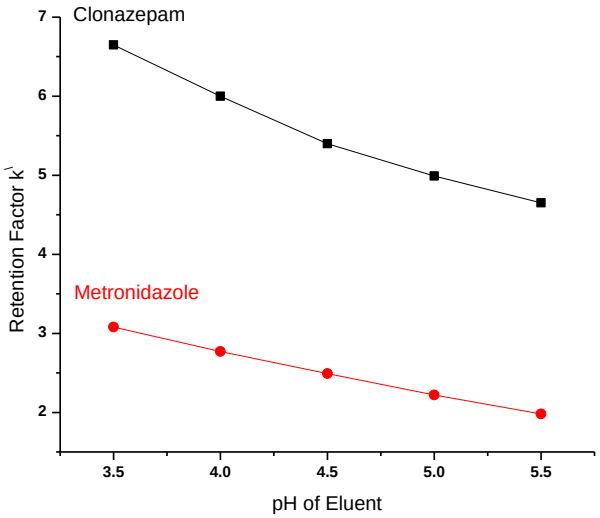

Figure 5. *The buffer pH effect on retention factor for MTZ and CLO.*

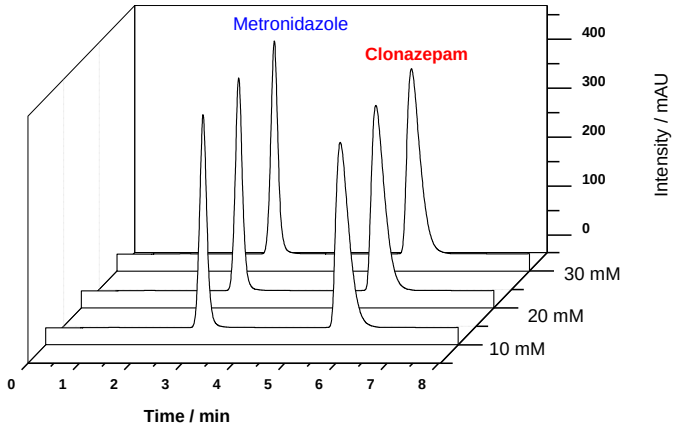

Figure 6. *Influence of buffer concentration on the chromatogram*

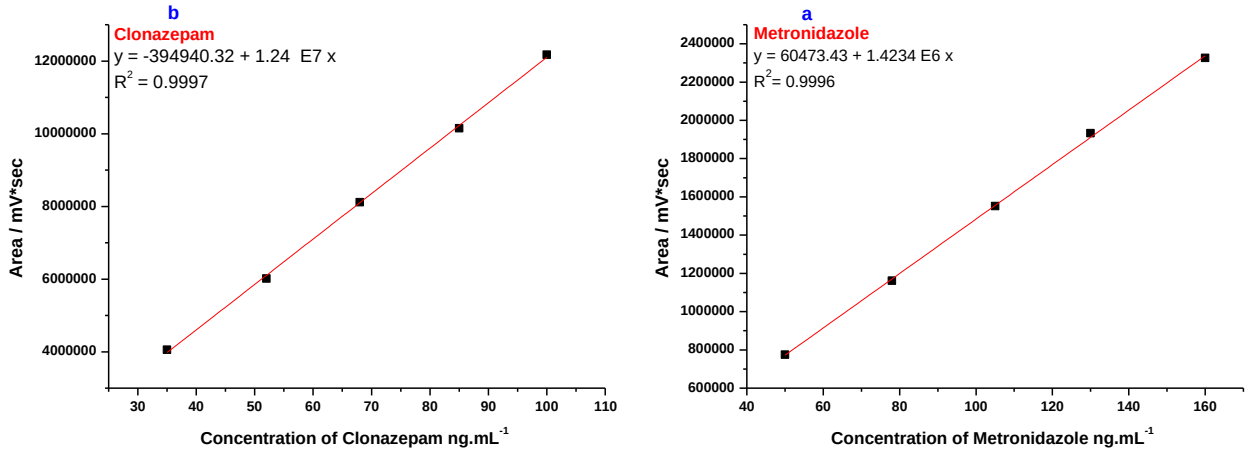

Figure 7. *Calibration graphs for CLO and MTZ.*

Table 1. *Analytical values of statistical treatments for determination of CLO and MTZ from the calibration graph*

Parameter	MTZ	CLO
Linear range (ppb)	50-160	35-100
Regression equation	y = 60473.43 + 1.4234 E6 x	y = -394940.32 + 1.24 E7 x
Coefficient of determination (r^2)	0.9996	0.9997
LOD (ppb)	4.24	3.06
LOQ (ppb)	14.15	10.21

Table 2. *The accuracy and precision of the proposed method for the determination of CLO and MTZ*

	intra-day n=5				inter-day n=5			
MTZ								
Present (ppb)	**Found (ppb)**	**%Rec.**	**% Erel.**	**%RSD**	**Found (ppb)**	**% Rec.**	**% Erel.**	**%RSD**
70	70.22	100.31	0.31	0.73	70.49	100.70	0.70	0.76
80	80.55	100.68	0.68	0.78	80.62	100.77	0.77	0.81
CLO								
70	69.5	99.28	- 0.72	0.44	70.05	100.07	0.03	0.55
80	79.86	99.82	- 0.18	0.50	79.97	99.96	- 0.04	0.59

Notes: %Rec.= Recovery percentage; % Erel. = Relative error percentage; %RSD=Relative Standard Deviation; Found (ppb)= The concentration found from the direct calibration graph

Table 3. *Implementation of the method of determination proposed of MTZ and CLO in pharmaceutical preparations*

Name of pharmaceutical	**Manufacturer**	**Present conc. (mg)**	**Found direct calb. (mg)**	**Rec %**	**RSD % n=5**	**RE %**
MTZ						
Negazole	Julphar, UAE	500	498.22	99.64	0.51	- 0.36
MEDAZOLE	SDI, Iraq	500	497.73	99.54	0.67	- 0.46
CLO						
Rivotril Hoffman-LaRoche	Hoffman-LaRoche, Switzerland	2.00	1.98	99.00	0.73	- 1.00
Rivotril Roche Farma, S.A	Roche Farma, Spain	2.00	1.97	98.50	0.33	- 1.50

Notes: Present conc. (mg)= The concentration of drug taken; Found direct calb. (mg)= The concentration found from the direct calibration graph.

Table 4. *The comparison of the suggested RP-HPLC method with conventional methods for MTZ and CLO measurements using t-test and F-statistical measures.*

Name of pharmaceutical	Proposed method Rec%	Standard method Rec% (Pharmacopeia, 2009)	t_{cal}	F_{cal}
MTZ				
Pure	100.495	99.87	0.97	1.67
Negazole	99.64	98.633		
MEDAZOLE	99.54	99.733		
CLO				
Pure	99.55	100.59	-0.42	4.47
Rivotril (2mg) Hoffman-LaRoche	99.00	98.55		
Rivotril (2mg) Roche Farma, S.A	98.50	98.81	*t_{tab} = 2.77	**F_{tab} =19.0

Theoretical values at 95% confidence limit, n1= n2 = 3.
**t = 2.776, where t has degrees of freedom = (n1 + n2 – 2) = 4*
***F = 19.0, where F has degrees of freedom = (n1 – 1) = 2, (n2 – 1) = 2*