Analysis of Chloramphenicol, Sulphamethoxazole and Sulfanilamide Drugs by Reversed- Phase High- Performance Liquid Chromatography with Fluorescent Detector

Lect. Dr. Tariq Y. Mahmoud, Lect. Dr. Aziz L. Jarallah and Assist. Lect. Isam S. Hamza

Dept. of Medical Lab. Technique –Al- Esraa University College, Baghdad / Iraq

E-Mail: dr.tariq@esraa.edu.iq

تحليل عقاقير الكلورامفينيكول، سلفاميثوكسازول والسلفوناميد بطريقة كروماتوكرافيا السائلة الطور العكسي عالية الأداء مع كاشف الفلورسنت

م. د. طارق ياسين محمود، م. د. عزيز لطيف جار الله و م. م. عصام شاكر حمزة

قسم تقنيات المختبرات المرضية، كلية الاسراء الجامعة – بغداد \ العراق

Abstract

This study proposed to analyze chloramphenicol (CPA), sulfamethoxazole (SMX) and sulfanilamide (SNA) drugs by reverse phase liquid chromatography method in the form of pharmaceutical preparations and synthetic mixture. For this method, column C18-ODS (25 cm × 4.6 mm) was used with an isocratic elution mobile phase composed of methanol and 2 % orthophosphoric acid, fluorescent detector (Ex= 310 nm, Em= 440 nm). It was found that the time of retention for CPA, SMX, and SNA 8.99 ± 0.02, 10.76 ± 0.2, and 8.21 ± 0.2 min. respectively. The suggested method has been validated for limit of detection, limit of quantitation, linearity, Accuracy and Precision. The method is sensitive with a detection limit (0.016 ppm, 0.044 ppm and 0.184 ppm) for CPA, SMX, and SNA respectively. The method accuracy 100.0% and the precision of this method that reflects by Coefficient of variation (CV) for the repeats is (0.002, 0.002 and 0.0025) for CPA, SMX, and SNA respectively. For routine analysis purposes, this method can monitor the drugs in the ingested pharmaceutical preparations.

Keywords: HPLC, Chloramphenicol, Sulphamethoxazole, Sulfanilamide.

المستخلص

اقترحت هذه الدراسة لتحليل العقاقير الكلورامفينيكول (CPA) والسلفاميثوكسازول (SMX) والسلفانيلاميد (SNA) بطريقة كروماتوكرافيا السائلة الطور العكسي والتي تكون على شكل مستحضرات صيدلانية وخليط صناعي. بالنسبة لهذه الطريقة ، تم استخدام العمود C18-ODS (25 سم × 4.6 مم) مع مرحلة شطف متغيرة النسب للطور المتحرك الذي تتكون من الميثانول و 2٪ من حمض الفوسفوريك ، كاشف الفلورسنت (الاثارة = 310 نانومتر ، الانبعاث= 440 نانومتر). وجد أن وقت الاحتجاز او الاستبقاء لـ 0.02 ± 8.99 (CPA) و و 0.2 ± 8.21 .(SNA) و (0.2 ± 10.76 SMX) دقيقة على التوالى. تم التحقق من صحة الطريقة المقترحة من خلال قياس حدود الكشف والمعادلة الخطية والضبط والدقة. الطريقة حساسة مع حد الكشف (0.016 جزء في المليون و0.044 جزء في المليون و 0.184 جزء في المليون) لـ CPA و SMX و SNA على التوالي. دقة الطريقة 100.0٪ ودقة هذه الطريقة التي تعكسها معامل التباين (CV) للتكرارات هي (0.002 ، 0.002 و 0.0025) لكل من CPA و SMX و SNA على التوالي. لأغراض التحليل الروتيني ، يمكن لهذه الطريقة مراقبة العقاقير في المستحضرات الصيدلانية التي يتم تناولها.

الكلمات المفتاحية: كروماتوغرافيا السائلة عالية الاداء ، كلورامفينيكول ، سلفاميثوكسازول ، سلفانيلاميد.

1. Introduction

Chloramphenicol (CPA) 2,2-dichlor-N-[(aR,bR)-b-hydroxy-a-hydroxymethyl-4-nitrophenethyl] acetamide produced by the growth of Streptomyces Venezuela or prepared synthetically_(The Pharmaceutical Codex,1979). It is an antibacterial drug helpful for the therapy of infections some bacterial (Vigh & Inczedy, 1976). It considered the first antibiotic synthesized widely. Chloramphenicol is active against broad spectrum micro-organisms. In ointment or eye drops Chloramphenicol is used to treat bacterial conjunctivitis (Vigh & Inczedy, 1976), (Fig. 1: a).

Sulphamethoxazole (SMX), Amino-N-(5-methylisoxazol-3-yl)-benzene sulfonamide is an antibiotic. It was applied to bacterial contagions such as infections of the urinary tract , bronchitis, and prostatitis and is active against both gram negative such as *E. coli & Listeria monocytogenes and positive bacteria* (Drug Bank,2015). (Fig. 1: b).

Sulfanilamide (SNA) ***p*-Amino benzene sulfonamide** is a sulfonamide antibacterial. Chemically, it is an organic compound containing aniline derivative with a sulfonamide group (Lipman,1993), (Fig.1: c).

Many methods have been used to analyze chloramphenicol in the presence of it is impurities by High-Performance Liquid (Sadana & Ghogarey,1991; Al-Rimawi and Kharoof, 2011 and Downy & Widart, 2013). HPLC methods for analysis of sulfanilamide (Sokolova & Chemyaev, 2005; Kilinc, *et al.*, 2009 and Waleed, *et al.*, 2013). Different methods of analysis have been reported for the determination of SMZ, including liquid

chromatography- mass spectrometry (LC–MS) (Herrera, *et al.*, 2013; Wang & Zhang, 2012 and Sorayya & Parvin, 2013).

The current work aims to find and validate the method of HPLC for the determination of three bacteriostatic antimicrobial compounds chloramphenicol, Sulfanilamide and Sulphamethoxazole in pure form, synthetic mixture preparations and pharmaceutical preparations. Method validation will be included accuracy, precision, specificity, linearity and range. The method can detect and appreciate the three drugs.

Developing a simple method of RP - HPLC for the determination of Chloramphenicol, Sulphamethoxazole and sulfanilamide individual and in mixture was the objective of this work.

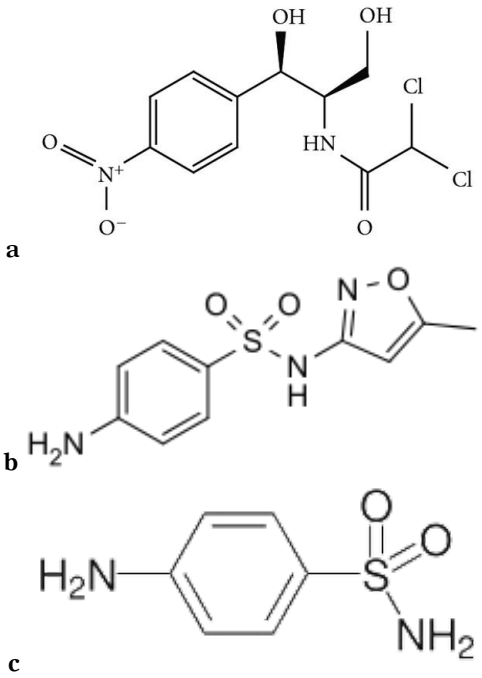

Fig. 1: (a) Structure of Chloramphenicol, (b) Sulphamethoxazole and (c) Sulfanilamide.

2. Materials and Methods

2.1. Chemicals

Methanol HPLC, Ethanol HPLC and orthophosphoric acid are from Merck and distilled water. Chloramphenicol, Sulfanilamide and Sulphamethoxazole drugs were provided by the State Company for the Pharmaceutical Industry and Medical Devices Samara – Iraq (SDI) in pure form (99.99%).

Reagents solutions: D.W (2 % orthophosphoric acid).

2.2. Apparatus

HPLC system (Sykam HPLC system, German) with an S- 1122 Solvent delivery system, an S-5200 sample injector, S-4011 column thermos controller, and fluorescent detector (Shimadzu – Japan) used, C18-ODS reverse-phase column with 250mm long and 4.6mm inner diameter (German). The Ezchrom Elite software is used. At room temperature the column is kept.

2.3. Standard Solutions and HPLC Conditions.

A solution of 2 % orthophosphoric acid was prepared by diluting 23.5 ml 85% orthophosphoric acid with water in 1000 ml volumetric flask to the mark.

Stock standard solutions of chloramphenicol sulfanilamide and sulphamethoxazole were made by dissolving 100 mg of each in methanol (100 ml) to get three solutions 1.0 mg per mL (1000 ppm) concentration of everyone's.

0.010 mg per ml (10 ppm) standard solutions chloramphenicol sulfanilamide and sulphamethoxazole were prepared by diluting 1 mL stock standard solutions of each one to 100 mL with methanol.

To prepare a solution for a mixture of the three compounds chloramphenicol, sulfanilamide and sulphamethoxazole at a concentration of 10 ppm for each one of them, 1 ml of the stock solution of a compound is added in the same volumetric flask (100 ml), and then completed the volume to the mark with methanol.

2.4. Preparation of sample solutions.

- Chloramphenicol and sulphamethoxazole in pharmaceutical.

  10 tablet or capsules was grinded and mixed well. To prepare 1000 ppm of sample solution, an amount of the powder equivalent to about 100 mg of chloramphenicol or sulphamethoxazole was accurately weighted and 50 mL of methanol was added for chloramphenicol or 50 mL of methanol was added for sulphamethoxazole. The solution was shaken and swirled before dilution to 100 mL with methanol in a volumetric flask. The un-dissolved materials were then filtered-off using Whatman filter paper No.41, and the first portion of the filtrate was discarded, before use. More dilute solutions were prepared freshly via diluting the stock solution with methanol as required, and the proposed method was applied for the determination of chloramphenicol content or sulphamethoxazole.

- Preparation of synthetic sulfanilamide drug sample.
  To prepare 1000 ppm solution of the synthetic drug, 100 mg was dissolved in 50 mL of methanol. The solution was then diluted with methanol in a 100 mL volumetric flask and filtered by the same manner as used for the preparation standard drug to obtain 1000 ppm.
- To prepare synthetic solution containing mixture of 500 ppm CPA, SMX and SNA dissolved in 10 ml methanol 0.05 g of each one and then diluted with Methanol in a 100 ml volumetric flask and filtered by the same method used to prepare the standard drug.

3. Results and Discussion

3.1 Method Development

C18-ODS reverse-phase column with 250mm long and 4.6mm inner diameter (German) was used for the analysis of chloramphenicol Fig.(2), Sulphamethoxazole Fig.(3) and Sulfanilamide Fig.(4), respectively. The chloramphenicol-sulfamethoxazole and sulfanilamide were separated from each other in a mixture. Regarding the mobile phase, a mixture of 2% orthophosphoric acid solution and methanol was used.

For the development of the isolation and symmetry of the peak, the mobile phase component was changed until the best composition was chosen; Isocratic elution has been employed in this study, where a decrease in flow rate was observed from the sixth minute until the end of the analysis to obtain better results, Table (1).

Table 1. Isocratic elution mobile phase: A: methanol, B: D.W (2 % orthophosphoric acid)

Initial	A %	B %	Flow rate ml/min
0 – 6	50	50	1.0
6 – 8	60	40	0.7
9 – 14	30	70	0.7

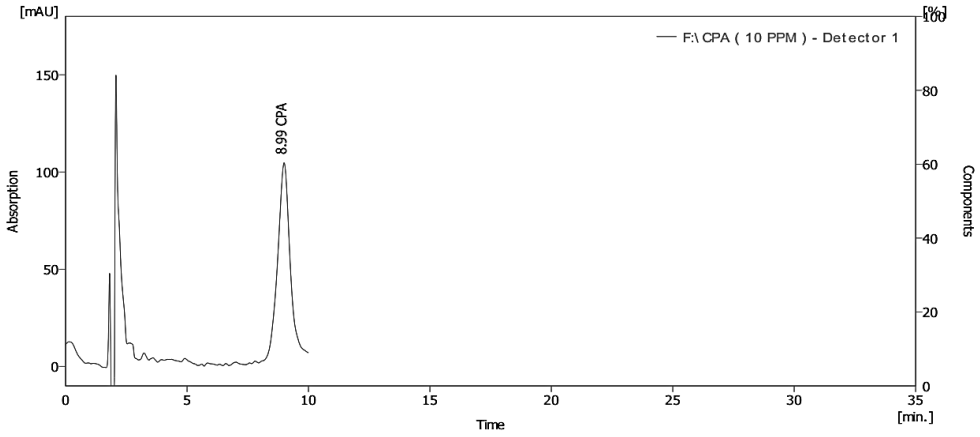

Fig. 2: Chromatogram for chloramphenicol analysis 10 ppm.

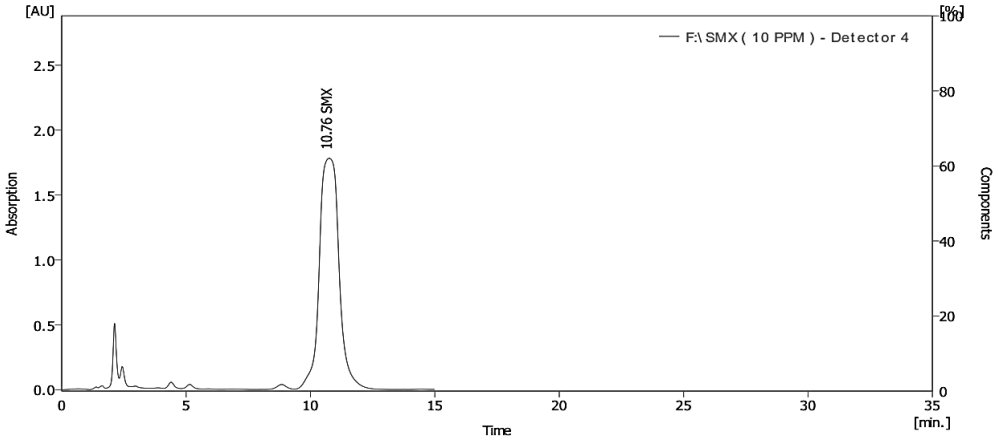

Fig. 3: Chromatogram for sulphamethoxazole analysis 10 ppm

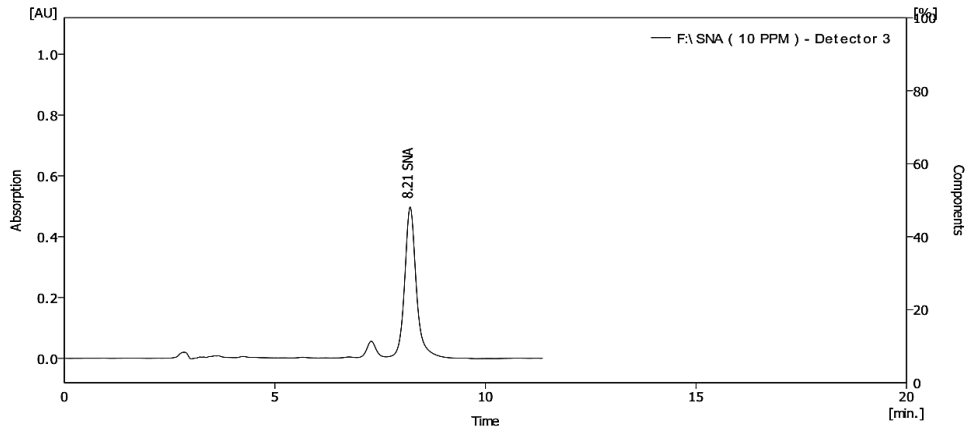

Fig. 4: Chromatogram for sulfanilamide analysis 10 ppm

At room temperature the column is kept during this study. It was noted that the separation process was not significantly affected by the change in column temperature. After this optimization, this method was used to separate chloramphenicol, sulfamethoxazole and sulfanilamide from each other in the same solution. Fig. (5). the result illustrate in Table (2) a suitable resolution has been obtained with a good separation.

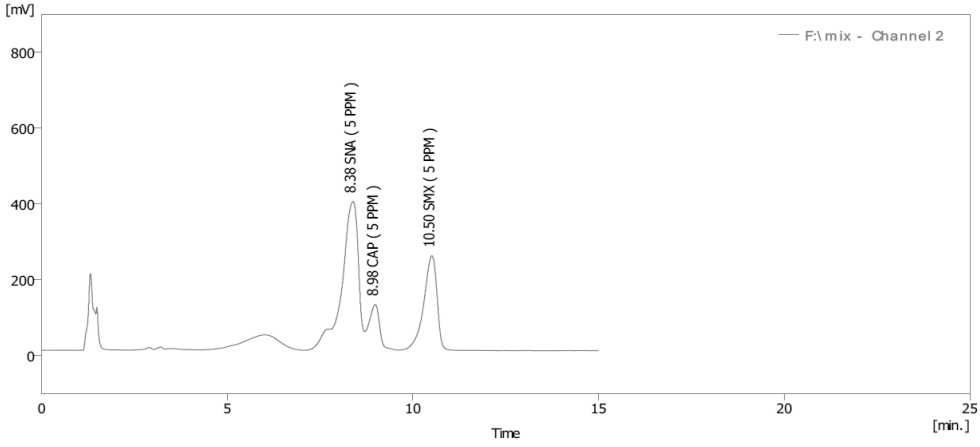

Fig. 5: Chromatogram for analysis of mixtures of SNA (5 ppm at 8.38 min), CPA, (5 ppm at 8.98 min) and SMX (5 ppm at 10.50 min).

Table 2: Result for analysis of drugs mixture.

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	8.383	161.995	26.108	7.5	14.6	0.11	SNA (5 PPM)
2	8.983	1452.439	92.858	67.0	52.1	0.28	CAP (5 PPM)
3	10.500	553.437	59.407	25.5	33.3	0.16	SMX (5 PPM)
	Total	2167.871	178.373	100.0	100.0		

3.2. Method Validation

After developing the method, Validate the present analysis method, for chloramphenicol, sulfanilamide and sulphamethoxazole have been performed as per the ICH guidelines for check determination, including linearity, range, precision, accuracy and specificity (Validation of Analytical Procedure, 2005).

3.2.1. Linearity and Range

A linear relationship is the ability of a method to give analysis results that are immediately proportional to the concentration of the substance being analyzed within a gotten range. Range is the domain-inter the lower and upper part of the concentration of the substance under analysis whose estimation has been demonstrated with accuracy, linearity and precision utilizing the written method. At least five concentrations are needed with some specific minimal ranges. The correlation coefficient (R2) is the acceptance criteria for a linearity of at least 0.990 for least-squares mode for line analysis. Standard solutions were prepared by diluting a certain stock standard volume (100 ppm) to obtain a concentration (2, 5, 10, 20, 30, 40 ppm). Three runs were performed for each concentration.

A calibration curve was recorded between peak areas versus standard concentration Figs. (6, 7, 8). The results shown in Table (3) illustrate the linear results of this method over the finite run.

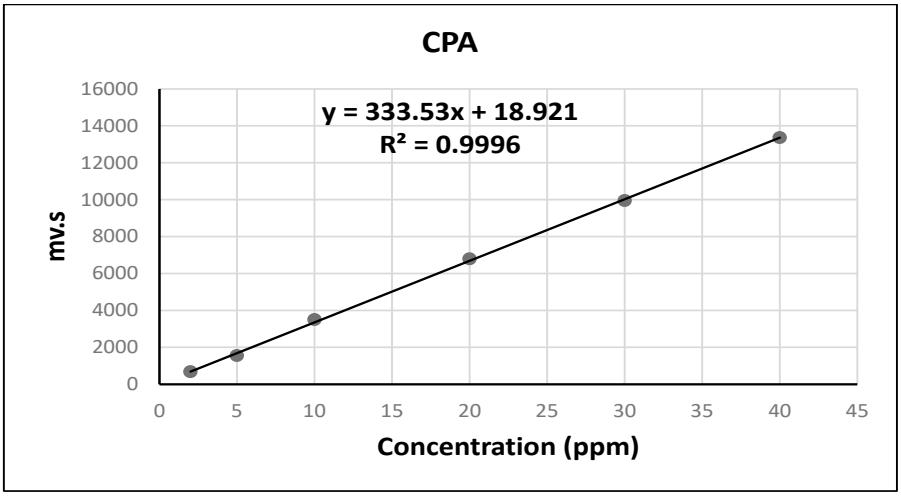

Fig. 6: Calibration curve was recorded between peak areas versus standard concentration (2, 5, 10, 20, 30, 40 ppm) for the determination of chloramphenicol under optimal conditions.

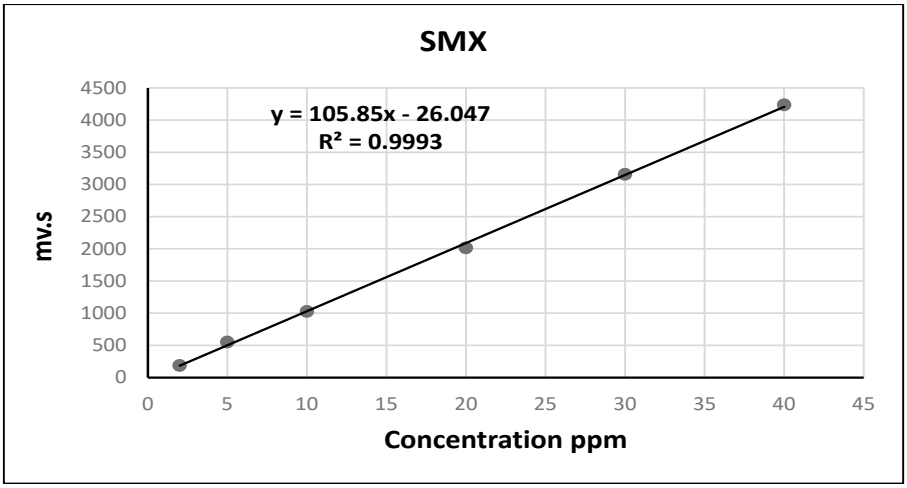

Fig. 7: Calibration curve was recorded between peak areas versus standard concentration (2, 5, 10, 20, 30, 40 ppm) for the determination of sulphamethoxazole under optimal conditions.

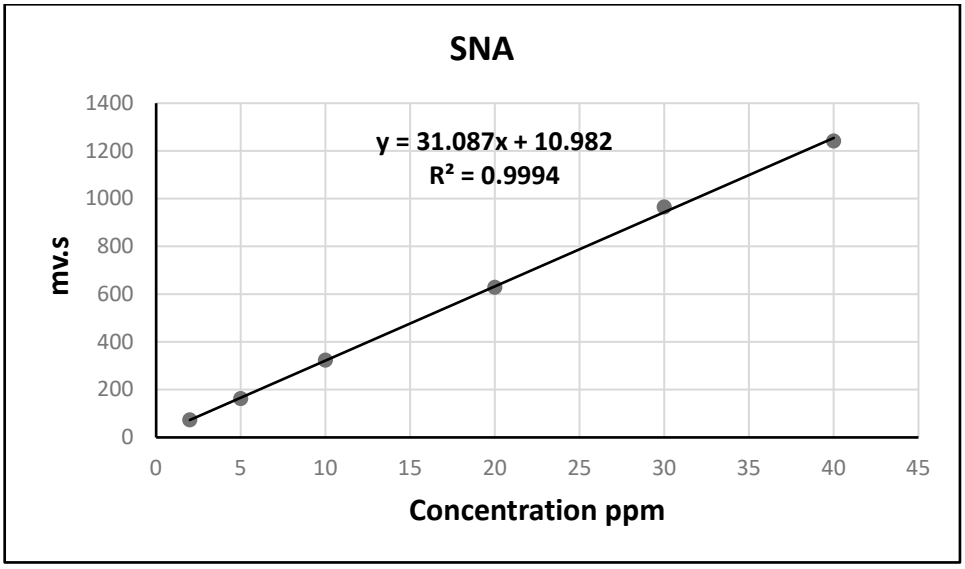

Fig. 8: Calibration curve was recorded between peak areas versus standard concentration (2, 5, 10, 20, 30, 40 ppm) for the determination of sulfanilamide under optimal conditions.

Table 3: Some statistical data to determine CPA, SMX and SNA via the recommended procedures.

	CPA	***SMX***	***SNA***
Regression equation	***y = 333.53x + 18.921***	***y = 105.85x - 26.047***	***y = 31.087x + 10.982***
Slop	333.53	105.85	31.087
R2	0.9996	0.9993	0.9994
Detection limit (µg/ mL)	0.016	0.044	0.184
Quantification limit (µg/ mL)	0.054	0.149	0.616

3.2.2. Solution Stability

Drug stability was checked using the proposed method by injecting fresh solutions of the substance to be analyzed using the system after 3, 12

and 24 hours. No drug degradation was noticed and no change in chromatogram peak area was seen during the study.

3.2.3. Accuracy and Precision

The accuracy of the analysis method measures how close a value which is either agreeable as a normal true value or an agreeable reference value. On the other hand precision described the agreement among several results obtained in the same way (Rockville,2007).

The accuracy and precision of the method were established by analyzing the pure drug at three concentration levels each for five replicates. Average recovery and CV were calculated for each level.

The results are shown in Table (4) which were satisfactory and showed good accuracy and precision by the proposed methods.

Table 4: Evaluation of accuracy and precision to determine CPA, SMX and SNA by the suggested method.

Materials	*Conc. (µg/mL)*		*Relative Error %*	*C.V %*
	Taken	*Found **		
CPA	10	10.070	0.700	0.0060
	20	20.300	1.500	0.0010
	30	30.212	0.706	0.0005
SMX	10	10.091	0.910	0.0040
	20	20.225	1.125	0.0040
	30	30.311	1.036	0.0005
SNA	10	10.055	0.550	0.0050
	20	20.198	0.990	0.0020
	30	30.257	0.856	0.0005

***Average of three determinations.**

3.3. Applications of this method

3.3.1 Application on pharmaceutical samples

Using the proposed chromatographic method, assay of CPA and SMX in its pharmaceuticals chloramphenicol capsule and eye drop Table (5), trimethoprim tablet and syrup were carried out Table (6). Satisfactory results were obtained for all two drugs in a good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets

Table 5: Application of the developed method to the chloramphenicol concentration measurements in pharmaceutical Sample.

Sample	***Conc. taken (µg.mL-1)***	***Conc.* found (µg.mL-1)***	***Recovery %***	***C.V* %***
PHENICOL (eye drop) SDI- Iraq	20.00	19.77	98.85	0.072
Chloramphenicol Capsule SDI- Iraq	20.00	20.04	100.2	0.070

***Average of three determination**

Table 6: Application of the proposed method to the SMZ concentration measurements pharmaceutical preparations.

Sample	***Conc. taken (µg.mL-1)***	***Conc.* found (µg.mL-1)***	***Recovery %***	***C.V* %***
Metheprim syrup SDI, Iraq	20.00	18.108	90.54	1.055
Methoprim tablet SDI, Iraq	20.00	19.56	97.8	1.036

***Average of three determinations**

3.3.2 Application in synthetic mixture sample

The application of suggested method for the purpose of determination of CPA, SMX and SNA in its synthetic mixture samples was successfully accomplished. The results are presented in Table (7), which show excellent recovery values that indicate the absence of any probable interference from each other or the excipients.

Table 7: Proposed method for determination of CPA, SMX and SNA in synthetic mixture.

Samples	*Concentration* Taken (µg/ mL)*	*Concentration* fond (µg/ mL)*	*Recovery %*	*C.V* %*
CPA in synthetic mixture sample	20.00	20.90	102.25	0.003
	30.00	31.01	101.68	0.0024
SMX in synthetic mixture sample	20.00	20.55	102.75	0.0023
	30.00	30.67	102.23	0.0036
SNA in synthetic mixture sample	20.00	20.32	101.6	0.0013
	30.00	30.72	102.4	0.0028

***Average of three determinations.**

Conclusion

Reversed- Phase High- Performance Liquid Chromatography with fluorescent Detector and isocratic elution mobile phase analytical method was developed and validated for the separation and determination of chloramphenicol, Sulphamethoxazole and sulfanilamide in a triple mixture. The obtained results confirmed the ICH guidelines for check identification include linearity, range, precision, accuracy and specificity. The suggested method is characterized by the ability to separate chloramphenicol, Sulphamethoxazole and sulfanilamide in synthetic mixture and in pharmaceutical preparations.

References

- "Sulphamethoxazole," Drug Bank, (2015).
- Al- Rimawi, A. and M. Kharoaf,(2011), "Analysis of Chloramphenicol and Its Related Compound 2-amino-1-(4- nitrophenyl) propane- 1,3- diol by Reversed-phase High Performance Liquid Chromatography with UV detection," Chromatography Research International.
- Douny, C. and J. Widart,(2013), "Determination of Choramphenicol in Honey, Shrimp, and Poultry Meat with liquid Chromatography-mass spectrometry," Vol. 6, pp. 1458-1465.
- Kilinc, E. B. Gumgum, C. Hamamci and F. Aydin, (2009),"Stability-indicating High Performance Thin Layer Chromatographic Determination of Sulfanilamide in Human Urine," Journal of Analytical Chemistry, Vol. 64, pp. 714-720.
- Lipman, A.,(1993), Martindale -the Extra Pharmacopoeia, Reynolds.
- Sadana, G. and A. Ghogare, (1991)."Simultaneous Determination of Choramphenicol and Benzocaine in Topical Formulations by High-performance Liquid Chromatography," Journal of Chromatography, Vol. 542, No. 2, pp. 515-520.
- Sokolova, L. and A. Chernyaev,(2005), "Reversed-phase HPLC Analysis of A mixture of Sulfanilamide Drug in Biological Fluids and Tissues," Pharmaceutical Chemistry Journal, Vol. 39, pp. 336-338.
- The Pharmaceutical CODEX, UK: British, (1979).
- Vigh G. and J. Inczedy,(1976), "Separation of Some Chloramphenicol Intermediates by High- pressure Liquid Chromatography," Journal of Chromatography, Vol. 116, No. 2, pp. 472-474.
- Waleed, M. N. Khaleel and G. Hadad,(2013), "Simultaneous Determination of 11 Sulfonamides by HPLC-UV and Application for Fast Screening of Their Aerobic Elimination and Biodegradation in A simple Test," Clean-soil Air Water, Vol. 41, No. 9, pp. 907-916.
- Herrera, A. J. Hernandez, M. Afonso, J. Palenzuela and M. Rodriguez, (2013),"Comparison Between Magnetic and Nonmagnetic Multi-walled Carbon Nanotubes-dispersive Solid-phase Extraction Combined with Ultra-high Performance Liquid Chromatography for the Determination of Sulfonamide Antibiotics in Water Samples," Talanta, Vol. 116, pp. 695-703.

- Wang, X. and Y. Zhang,(2012), "Simultaneous Determination of Sulphamethoxazole and Trimethoprim in Rat Plasma by LC-ESI-MS and Its Application to A Pharmacokinetic Study," Journal of Liquid Chromatography and Related Technologies, Vol. 35, No. 7, pp. 951-962.
- Sorayya, A. and G. Parvin,(2013), "Simultaneous Determination of Sulfamethoxazole and Phthalazine by HPLC and Multivariate Calibration Methods," Iranian Journal of Chemistry and Chemical Engineering-international , Vol. 32, No. 2, pp. 1-8, 2013.
- "Validation of Analytical Procedures,"(2005), in International Conference on Harmonization ICH, Europe.
- Rockville, A. (2007),"Pharmacopoeia National Formulary, Validation of Compendia Methods," USA, pp. 1225.