ISSN-1996-918X

Pak. J. Anal. Environ. Chem. Vol. 24, No. 2 (2023) 135 – 144

http://doi.org/10.21743/pjaec/2023.12.02

Green Spectrophotometric Method for Determination of Mesalazine Drug by Oxidative Coupling Reaction with Phenoxazine

Abdussamed Mohammed Ali Saeed[*1], Intisar Adil Shihab Al-Hammoodi[2], Mohammed Salim Al-Enizzi[2] and Suhair Mahfoodh Salih[2]

[1]*Section of Basic Science, College of Agriculture and Forestry, University of Mosul, Mosul, Iraq.*

[2] *Department of Chemistry, College of Education for Girls, University of Mosul, Mosul, Iraq.*

Corresponding author Email: abdmas74@uomosul.edu.iq

Received 06 January 2023, Revised 10 October 2023, Accepted 16 November 2023

Abstract

A novel green, straightforward, and precise spectrophotometric method for determination of mesalazine drug has been developed. This method relies on an oxidative coupling reaction between mesalazine and the environmentally friendly phenoxazine reagent in an acidic medium within the presence of potassium iodate as an oxidizing agent. The resulting colored product exhibits maximum absorbance at 549 nm. The calibration graph was rectilinear over the range of 0.2-30 µg mL^{-1} (1.31×10^{-6}-1.96×10^{-4} mol L^{-1}) with determination Coefficient (R^2) of 0.9994 and molar absorptivity of 4.8×10^{3} L mol^{-1} cm^{-1}. The limits of detection and quantitation were found to be 0.191 and 0.638 µg mL^{-1}, respectively. The average recovery percentage was 100.9 with a relative standard deviation less than 3.1. The proposed method was effectively applied to determine mesalazine in tablet dosage forms from three different sources, yielding highly satisfactory results that closely align with the standard method outlined in the British Pharmacopeia.

Keywords: Green spectrophotometric method; Mesalazine, Oxidative coupling reaction, phenoxazine.

Introduction

The concept of green or sustainable chemistry has emerged as a relatively new paradigm over the last three decades, with a primary focus on redesigning chemical processes to use or produce less harmful substances concerning human health and the environment [1]. Green analytical chemistry plays a crucial role in monitoring pollution content. Today, analysts bear a significant responsibility to innovate new green analytical methods as much as possible without affecting sensitivity, accuracy and precision. Achieving this concept involves considering various strategies, one of which includes the use of less environmentally harmful chemical reagents and solvents [2]. Recent innovations in analytical methods have proposed alternatives such as replacing organic solvents with surfactants as green solvents [3-5]. In this paper, a new green spectrophotometric method was suggested for the determination of mesalazine (MZN) using phenoxazine (PXN) reagent. The latter (Fig. 1A) was first synthesized by Bernthsen in 1887 [6]. It is considered a green reagent [7] and a core component of the antitumor drug dactinomycin [8,9]. Given its significance, various studies have been conducted to

synthesize derivatives of PXN and explore their pharmaceutical, biological, or industrial applications [10-13].

MZN, also known as 5-aminosalicylic acid or mesalamine (Fig. 1B) [14] is used to treat ulcerative colitis and Crohn's disease [15] collectively referred to as inflammatory bowel diseases [9]. According to the reviewed literature, several methods have been reported for MZN determination, including spectrophotometric [16-20], electrical [21-23] and HPLC methods [24,25]. Our proposed method is based on the oxidative coupling reaction of MZN with PXN in an acidic medium in the presence of potassium iodate as an oxidizing agent. The resulting reddish-purple product exhibits maximum absorbance at 549 nm. We conducted a comprehensive study of all experimental parameters. The method was successfully applied to the pharmaceutical preparation of MZN in tablet form. Furthermore, The suggested method was evaluated against the British Pharmacopeia method, yielding highly satisfactory results.

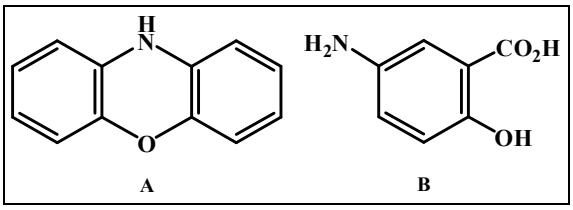


Figure 1. **Chemical structure of phenoxazine (A) and mesalazine(B)**

Material and Methods

Apparatus

All spectrophotometric measurements were accomplished by using a Spectro UV-VIS AUTO spectrophotometer from Labomed Inc.- USA equipped with a standard 1 cm path-length fused silica cuvette. The weighing processes were carried out by ADAM electronic balance from Adam Equipment-China. A Genlab Limited-UK water bath was used for heating optimization tests. An EUTECH-Singapore pH meter was used for pH measurements.

Chemicals and Reagents

All chemicals were of analytical grade products. Mesalazine in pure form was obtained from a company of SDI-Iraq. Phenoxazine and potassium iodate were obtained from Sigma-Aldrich-USA. Hydrochloric acid was obtained from Scharlau-Spain. All solutions were prepared by using distilled water. A 200 µg mL^{-1} stock solution of MZN in pure form was prepared by dissolving 0.020 g of pure MZN powder in 10 mL absolute ethanol with slight heating, then diluting to 100 mL with distilled water. Other working standard solutions 100, 50 and 20 µg mL^{-1} were obtained by the appropriate dilution of stock solution with distilled water. PXN stock solution (6×10^{-3} mol L^{-1}) was prepared by dissolving 0.1099 g in 100 mL absolute ethanol in a calibrated flask of 100 mL. Other working solutions were obtained by the appropriate dilution of stock solution with absolute ethanol. Hydrochloric acid solution was prepared by dilution of an appropriate volume of concentrated acid (Sp.gr. 1.19 g cm^3) with distilled water to 100 mL in a volumetric flask. The final solution was calibrated with a standard solution of sodium hydroxide. Potassium iodate stock solution (6×10^{-2} mol L^{-1}) was prepared by dissolving 1.2840 g in 100 mL distilled water in the calibrated flask of 100 mL. Working standard solutions were obtained by suitable dilution of stock solution with distilled water.

Procedure for Calibration Graph

According to the optimized recommended procedure, a calibration graph was constructed (n=5) by transferring an increasing volume of MZN solution into a series of 10 mL volumetric flasks to cover the determination range. Then 0.6 mL of PXN

reagent (2×10^{-3} mol L^{-1}) was added to each. 0.5 mL of HCl acid (1×10^{-1} mol L^{-1}) was added to all flasks. Finally, 1 mL of potassium iodate solution (3×10^{-2} mol L^{-1}) was added. The flasks were completed with distilled water, the flask was complete to the appointed mark. they were mixed well. After a five-minute standing time, an absorbance was measured at 549 nm.

Procedure for formulations

Three different brands of MZN formulation as tablets were obtained from a local market. Tablet I: Pentasa tablet 500 mg from FERRING Pharmaceuticals-Turkey, batch no. R08312A, tablet II: Salmimet tablet 500 mg from Bilim Pharmaceuticals-Turkey, batch no. 005A and tablet III: Mesacol tablet 400 mg from UNIPHARMA-Syria, batch no. 19DEFG 5. For each brand, ten tablets were weighed and crushed into a smooth powder. A part the ten tablets powder was weighed and dessolved in a beaker with 25 mL absolute ethanol and slight heating, then transferred into a 1000 mL volumetric flask and diluted to the mark with distilled water. After shaking well, the solution was filtered through No. 42 Whatman filter paper. The obtained solution (500 µg mL^{-1} for tablets I and II, or 400 µg mL^{-1} for tablet III) was used to prepare other solutions for the suggested determination method by appropriate dilution.

Results and Discussions

The major goal of the green chemistry principle is to encourage analysts to think and find out new analytical procedures for different component determination. These procedures vary respectively due to their uniqueness. One of them is using green reagents. In this paper, a new spectrophotometric method was suggested for the determination of MZN. The method uses PXN as a green reagent. The latter has different applications and research in various fields, but there is a lack of focus on it as an analytical reagent. This seems very interesting to use it for MZN determination via oxidative coupling reaction in an acidic medium by using potassium iodate as an oxidizing agent.

All the optimization steps were accomplished on 15 µg mL^{-1} (1.5 mL of 100 µg mL^{-1}) MZN. A preliminary study was performed by adding 0.5 mL PXN (1.5×10^{-3} mol L^{-1}), 1 mL HCl (8×10^{-2}mol L^{-1}) and 1 mL $NaIO_4$(2×10^{-2}mol L^{-1}) to 10 mL calibrated flask. A preliminary absorption spectrum was obtained after dilution and waiting for 5 min (Fig. 2).

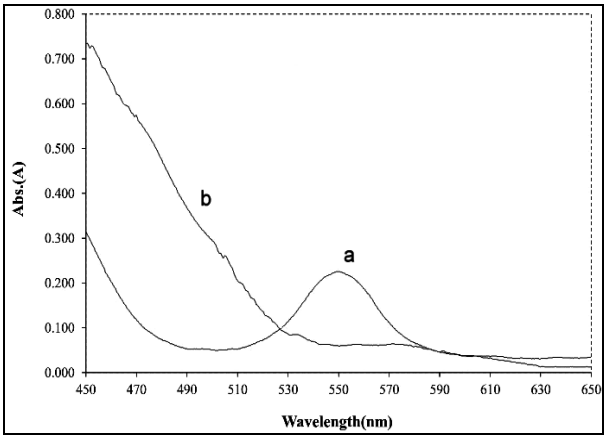


Figure 2. **Preliminary spectrum of 15 µg mL^{-1} MZN against blank (a) and blank against distilled water (b)**

Effect of Oxidizing Agent

First of all, the effect of oxidizing agent type was explored. Five types of these agents were tested (1 mL of 2×10^{-2} mol L^{-1} was added). These included n-bromo-succinimide, sodium nitroprusside, potassium dichromate, potassium iodate and sodium metaperiodate. The best absorbance (0.251) was achieved with potassium iodate. In addition, different concentrations of the latter were tested. The best one was 3×10^{-2} mol L^{-1} (Fig. 3A). Furthermore, the ideal volume of this concentration was 1 mL (Fig. 3B).

Effect of Acids and Buffers

The acidity of the reaction medium is very important in oxidative coupling reactions. No color was obtained in the absence of an acidic medium. Five types of acids were explored (1 mL of 8×10^{-2} mol L^{-1} was added). These acids included hydrochloric, sulfuric, nitric, phosphoric and acetic. However, hydrochloric acid was found to be the best one for the reaction medium with the best absorbance (0.304). It was the same one used in the previous steps. Moreover, different concentrations of hydrochloric acid were tested. The ideal concentration was 1×10^{-1} mol L^{-1} (Fig. 3C). In addition, the best volume of the latter was 0.5 mL (Fig.3D). In a related context, the addition of different types of buffer solutions instead of HCl was studied in the range of pH 1-3. This range was around the final pH of the reaction solution (2.2) in the case of using HCl acid from the previous step. The results in Table 1 show the bad effect of buffers on absorbance therefore, buffer solutions were excluded.

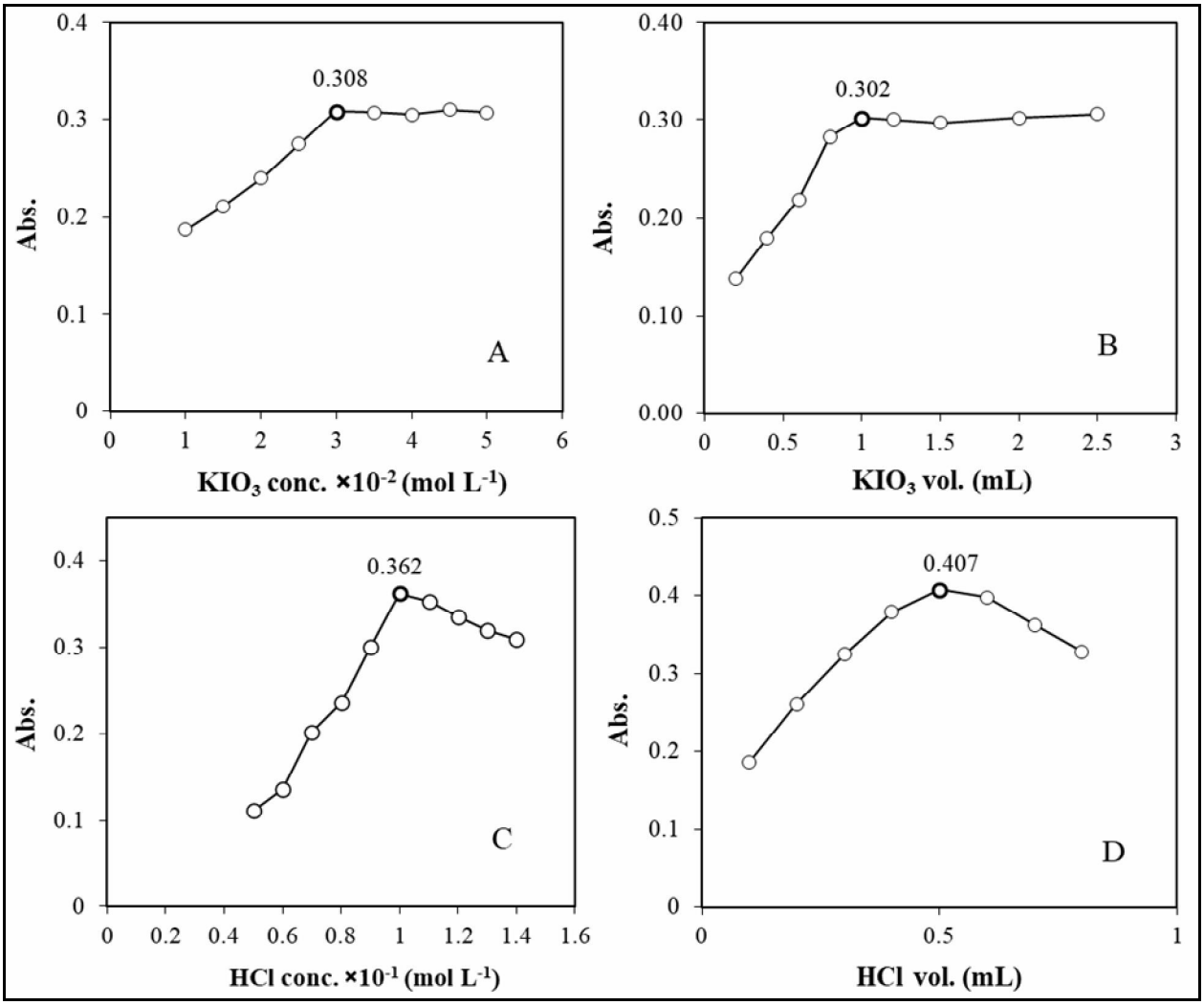


Figure 3. Effect of KIO_3 concentration (A), effect of KIO_3 (3×10^{-2} mol L^{-1}) volume (B), effect of HCl concentration (C) and effect of HCl (1×10^{-1} mol L^{-1}) volume (D)

Table 1. **Effect of buffer solutions.**

Buffer solution	pH	Abs	Final pH
KCl, HCl	1	0.179	1.8
	2	0.154	2.4
	3	0.147	3.4
KH Phthalate, HCl	1	0.226	1.9
	2	0.211	2.6
	3	0.148	3.6
Citric acid, NaOH	1	0.195	1.9
	2	0.183	2.8
	3	0.156	3.7

* 1.0 mL of buffer solution added

Effect of PXN Reagent

The effect of PXN reagent was studied in both cases of concentration and volume. The effect of PXN concentration in the range of 1×10^{-3}-3×10^{-3} mol L^{-1} was tested. The best concentration which gave the highest absorbance (0.461) was 2×10^{-3} mol L^{-1}. The effect of the latter volume was also explored. It is noticed that the increase of PXN volume resulted in an increase of absorbance, but it also led to an increase in the absorbance of blank against water (Table 2). It seems appropriate to adopt 0.6 mL as an ideal volume of PXN for the determination procedure.

Table 2. **Effect of PXN volume.**

Vol. of PXN (2×10^{-3} mol L^{-1})	Abs.	
	Sample vs. blank	Blank vs. water
0.2	0.135	0.031
0.3	0.293	0.077
0.4	0.364	0.106
0.5	0.467	0.171
0.6	0.504	0.223
0.7	0.543	0.453
0.8	0.571	0.511
0.9	0.593	0.612

Effect of Reaction Time and Temperature

The effect of reaction time over different temperatures was studied via periodic absorbance measurements according to the times and temperatures shown in Table 3. The latter shows the room temperature (20 ^{o}C ±1) was the best one with 5 min standing time before measurement. While at the lower temperature (10 ^{o}C) the reaction needs approximately one hour to complete.

Table 3. Effect of time and temperature.

Time (min.)	Abs.			
	10°C	R.T*	30°C	40°C
5	0.444	0.509	0.451	0.408
10	0.450	0.507	0.450	0.406
15	0.466	0.506	0.448	0.403
20	0.471	0.507	0.447	0.405
30	0.480	0.509	0.439	0.407
40	0.502	0.506	0.446	0.402
50	0.507	0.505	0.447	0.405
60	0.508	0.507	0.445	0.402
120	0.502	0.504	0.440	0.393
Overnight	---	0.367	---	---

* Room temperature (20 ^{o}C ±1)

Effect of Surfactants

The study of surfactants effect is of much interest in green chemistry. These compounds have many advantages on the determination procedure if they were harmonized with reaction kinetic. Therefore, the effect of surfactants on absorbance was explored. Three different types were tested. These included anionic: sodium dodecyl sulfate (SDS), cationic: cetyl tetra ammonium bromide (CTAB) and non-ionic: polyoxyethylene sorbitan monopalmitate (Tween 40). 1 mL of each was added (1% of Tween 40 and 0.5% of SDS and CTAB). No significant enhancement on absorbance was noticed.

Order of Addition Effect

The sequence of the reaction components addition was studied. The order that included the addition sequence of MZN, PXN, HCl and KIO_3 gave the best absorbance (0.505). This sequence was followed in the previous steps. While the sequence of KIO_3, HCl, PXN and MZN gave the lowest absorbance (0.278).

Method Validation

After the optimization study, final absorbance spectrum of MZN determination was scanned as illustrated in Fig. 4.

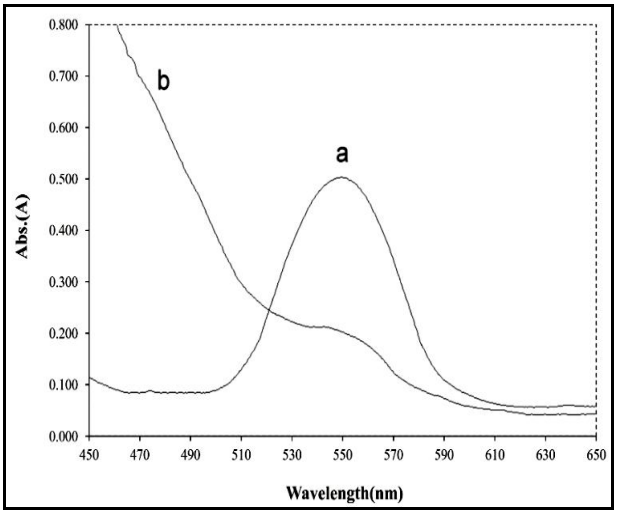


Figure 4. **Optimized spectrums of 15 µg mL^{-1} of MZN against blank (a) and blank against distilled water (b)**

The standard calibration graph of MZN determination was constructed under the optimal conditions as described above from linear regression of absorbance against MZN concentration (Fig. 5). The linearity was excellent in the range of 0.2-30 µg mL^{-1}. Molar absorptivity was 4.8×10^3 L mol^{-1} cm^{-1}. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.191 and 0.638 µg mL^{-1}, respectively. These values showed the high sensitivity of the presented method. The standard deviation of slope and intercept were 0.00028 and 0.00465, respectively.

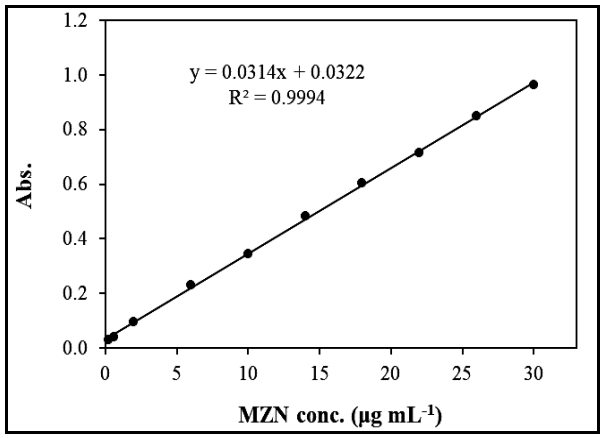


Figure 5. **calibration graph (n=5) of MZN according to the suggested method**

Precision and Accuracy

The precision and accuracy of the suggested method were evaluated by measuring five duplicate analyses on pure MZN solutions at four different concentrations. Table 4 reveals the average recovery (%) and relative standard deviation (RSD) which indicates that the presented procedure is accurate and precise enough.

Table 4. **Precision and accuracy of presented method.**

Taken amount (µg mL^{-1})	Recovery* (%)	Average recovery (%)	RSD %
2	101.9	100.9	1.139
10	99.7		1.148
18	101.2		3.055
26	100.2		2.009

*Average of five determinations

Effect of additives

In order to test method selectivity, the effect of some common excipients on the absorbance of 15 µg mL^{-1} MZN was studied. The excipients solutions were added in four different amounts for each. The listed results shown in Table 5 indicate that no significant effect of the additives on the estimation method suggested.

Table 5. **Effect of additives on recovery (%) of 15 µg mL^{-1} of MZN.**

Excipients	Recovery (%) of 15 µg MZN per µg of excipients added		
	300	600	1000
Acacia	102.51	99.53	96.14
Glucose	97.62	100.17	96.14
Fructose	100.81	96.35	95.29
Sucrose	96.77	102.29	94.86
Starch	95.71	95.07	99.53
Sodium chloride	98.68	95.71	95.50
Potassium chloride	96.14	97.41	100.38
Magnesium carbonate	104.42	98.05	105.05

Stoichiometry and Stability of the Reaction Product

It is important to study the nature of the reaction and the stability of colored products. Continuous variation (Fig. 6A) and mole ratio (Fig. 6B) methods were applied to determine the reaction stoichiometry [26]. Both method plots show that the mole ratio is 1:1 (MZN:PXN). The stability constant of colored product was calculated by application of 1:1 stability constant equation [26]:

$$K_{st} = \frac{1-\alpha}{\alpha^2 C}$$

Where C is the concentration of the reaction product which is similar to MZN concentration (mol L^{-1}), α=degree of dissociation and it is equal to [A_m-A_s/A_m], A_m and A_s were performed by measuring three different volumes of MZN (0.5, 1 and 1.5 mL of 5×10^{-4} mol L^{-1} solution) once in the presence of same volumes of 5×10^{-4} mol L^{-1} PXN (A_s value) and once in the presence of optimal volume and concentration of PXN (0.6 mL of 2×10^{-3} mol L^{-1} solution) (A_m value). The stability constant values for three volumes of MZN were 1.48x10^5, 8.02x10^4 and 5.65x10^4 mol L^{-1}, respectively, with an average of 9.47x10^4 mol L^{-1}. The latter value revealed good stability of colored product of the proposed method.

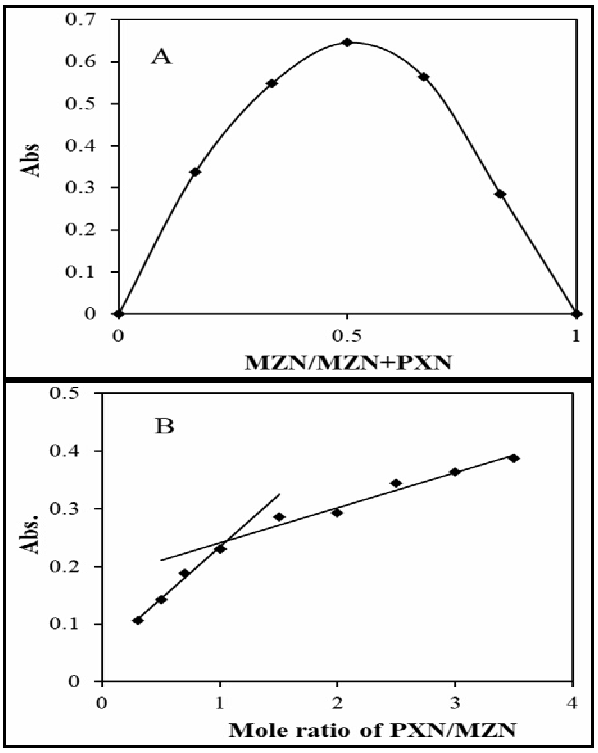
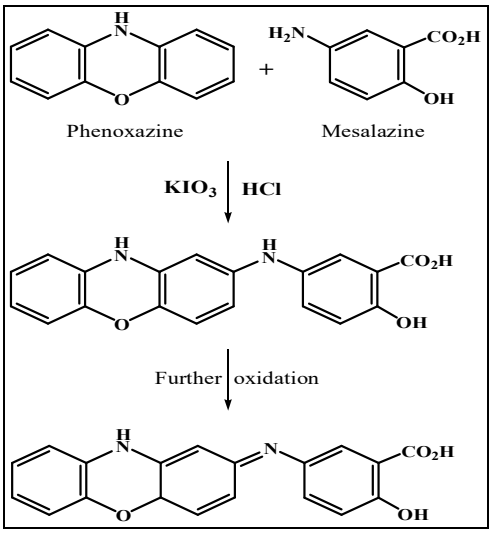


Figure 6. **Continuous variation method (Job's method) (A) and mole ratio method (B)**

Suggested Reaction Mechanism

After the optimization and stoichiometry experiments, it is useful to predict a suitable mechanism of the reaction. Scheme 1 shows the expected mechanism [27, 28].



Scheme 1. **Expected mechanism of the suggested method reaction**

Table 6. **Application of suggested method on pharmaceutical formulations and comparison with BP method.**

F-exp	t-test	Recovery** (%) BP method	MZN content found (mg)	Average recovery (%)	Recovery* (%)	Amount taken (µg mL^{-1})	Certified value (mg)	Formulation
0.023	0.015	100.43	498.41	100.33	99.68	2	500	Tablet I
			501.27		100.25	10		
			505.31		101.06	18		
			504.29		100.86	26		
1.694	0.482	99.13	493.63	98.95	98.73	2	500	Tablet II
			496.50		99.30	10		
			494.16		98.83	18		
			504.41		100.88	26		
0.449	0.634	99.82	398.73	100.99	99.68	2	400	Tablet III
			404.84		101.21	10		
			408.35		102.09	18		
			403.92		100.98	26		

* Average of five determinations, **Average of four determinations

Determination of Pharmaceutical Formulation

Due to the procedure, three different pharmaceutical formulations of MZN tablets were considered. Table 6 reveals that the reached values are in good agreement with certified values. This indicates the accuracy and precision of the suggested method.

Evaluation of the Proposed Method

Evaluation of the suggested method was performed by statistically comparing [29] its results with the standard procedure of British Pharmacopeia [14]. Table 6 shows t and F test values. These values were the smallest ones than their statistically recorded values at %95 confidence level (2.45 and 9.28, respectively). As for the precision between the suggested and the standard method, it can be concluded that there is no significant difference.

Furthermore, it seems useful to make a comparison of some analytical parameters between the suggested method and others in the literature which used oxidative coupling reaction to determine MZN (Table 7).

Table 7. **A comparison between suggested method and some published methods for the determination of MZN via oxidative coupling reaction.**

Parameters	Suggested method	Aziz and Sultan [18]		Shehab and Muhammed [19]
		A	*B*	
Reagent	PXN	m-aminop-henol	2,6-dihydroxy-benzoic acid	PXN
Linearity range (µg/mL)	0.2-30	1.25-30	0.5-12.5	0.1-11
Molar absorptivity (L.mol^{-1}.cm^{-1})	4.8×10^3	3.6×10^3	7.7×10^3	2.1×10^4
(R^2)	0. 9994	0.9951	0.9971	0.9978

Conclusion

The trend of green chemistry and the risks of high levels of pollution encourage analysts to develop new environmental and friendly determination methods. These were focused on the principle of using harmless materials, which included environmentally friendly solvents and reagents. The green reagent of phenoxazine promotes this concept. This reagent is suitable to estimate mesalazine drug with good sensitivity and accuracy. The suggested method is simple and free from experimental details such as extraction or heating steps. The good results from the

application of the presented method on tablets suggest to use of it for routine quality control tests on bulk and pharmaceutical formulations.

Acknowledgement

Authors would like to express their gratitude to the University of Mosul and the College of Agriculture and Forestry for the support and facilities to carry out this work.

Conflict of Interest

All the authors of this manuscript declare that there is no conflict of interest related to the research work presented in this manuscript.

References

1. P. T. Anastas, *Crit. Rev. Anal. Chem.*, 29 (1999) 167. https://doi.org/10.1080/10408349891199356
2. C. Turner, *Pure Appl. Chem.*, 85 (2013) 2217. http://dx.doi.org/10.1351/PAC-CON-13-02-05.
3. R. A. Khalil and A. M. A. Saeed, *J. Chin. Chem. Soc.,* 54 (2007) 1099. https://doi.org/10.1002/jccs.200700157.
4. R. A. Khalil and R. Z. Al-Khayat, *Phys. Chem. Liq.*, 46 (2008) 34. https://doi.org/10.1080/00319100601084993.
5. E. S. Thanoon and A. M. A. Saeed, *Egypt. J. Chem.*, 64 (2021) 3451. https://doi.org/10.21608/EJCHEM.2021.43857.2923.
6. A. Bernthsen, *J. Org. Chem.*, 20 (1887) 942. https://doi.org/10.1002/cber.188702001214.
7. R. A. AL-Okab, M. S. A. Galil and A. N. Al-Hakimi, *Pharm. Anal. Acta,* 9 (2018) 1000584. https://doi.org/10.4172/2153-2435.1000584.
8. J. Koshibu-Koizumi, M. Akazawa, T. Iwamoto, M. Takasaki, F. Mizuno, R. Kobayashi, A. Abe, A. Tomoda, M. Hamatake and R. Ishida, *J. Cancer Res. Clin. Oncol.,* 128 (2002) 363. https://doi.org/10.1007/s00432-002-0352-3.
9. B. G. Katzung, Basic & clinical pharmacology, 14th ed., McGraw-Hill Education, USA (2018). https://accessmedicine.mhmedical.com/content.aspx?bookid=2249&sectionid=175215310
10. J. G. Zorrilla, C. Rial, D. Cabrera, J. M. Molinillo, R. M. Varela and F. A. Macías, *Molecules,* 26 (2021) 3453. https://doi.org/10.3390/molecules26113453.
11. F. N. Ibeanu, E. A. Onoabedje, A. Ibezim and U. C. Okoro, *Med. Chem. Res.*, 27 (2018) 1093. https://doi.org/10.1007/s00044-017-2131-3.
12. M. Kientz, G. Lowe, B. G. McCarthy, G. M. Miyake, J. Bonin and M. Robert, *Chem. Photo Chem.*, 6 (2022) e202200009. https://doi.org/10.1002/cptc.202200009.
13. L. Li, X. Dai, X. Liao, X. Zang, J. Huang, H. Zhang, X. Yin and Y. Hong, *Sol. Energy*, 225 (2021) 173. https://doi.org/10.1016/j.solener.2021.07.030.
14. British Pharmacopoeia, vol II, The Stationery Office, London (2022). https://www.pharmacopoeia.com
15. G. R. Garg and S. Gupta, Review of pharmacology, 9th ed., Jaypee Brothers Medical Publishers (P) Ltd, New Delhi, India (2015). https://www.abebooks.com/9789351528876/Review-Pharmacology-9ed-2015-Sparsh-9351528871/plp
16. A. A. Elbashir, F. A. A. Abdalla and H. Y. Aboul-Enein, *Lumin.*, 30 (2015)

1250. https://doi.org/10.1002/bio.2888.
17. M. M. Morcoss, N. S. Abdelwahab, N. W. Ali and M. T. Elsaady, *Chem. Pharm. Bull.*,64 (2016) 1268. https://doi.org/10.1248/cpb.c16-00143.
18. A. T. Aziz and S. H. Sultan, *Baghdad Sci. J.,* 16 (2019) 1010. http://dx.doi.org/10.21123/bsj.2019.16.4(Suppl.).1010.
19. A. A. Shehab and D. H. Muhammed, *Sys. Rev. Pharm,*. 11 (2020) 922. http://dx.doi.org/10.31838/srp.2020.6.130.
20. M. S. Al-Enizzi, I. A. Shihab Al-Hammoodi and A. M. A. Saeed, *Egypt. J. Chem*., 65 (2022) 1561. https://ejchem.journals.ekb.eg/article_269664.html
21. A. B. Teradale, S. D. Lamani, P. S. Ganesh, B. E. K. Swamy and S. N. Das, *Sens. Bio-Sens. Res.*, 15 (2017) 53. https://doi.org/10.1016/j.sbsr.2017.08.001.
22. B. Nigović, A. Mornar, E. Brusač and M.-L. Jeličić, *J. Electroanal. Chem.*, 851 (2019) 113450. https://doi.org/10.1016/j.jelechem.2019.113450.
23. E. Sohouli, M. S. Karimi, E. M. Khosrowshahi, M. Rahimi-Nasrabadi and F. Ahmadi, *Measurement*, 165 (2020) 108140. https://doi.org/10.1016/j.measurement.2020.108140.
24. B. Ceylan, E. K. Tekkeli and C. Önal, *J. Fluoresc.*, 32 (2022) 319. http://hdl.handle.net/20.500.12645/29669.
25. V. Tsamis, E. Tsanaktsidou, C. Karavasili, C. K. Zacharis, N. Bouropoulos, D. G. Fatouros and C. K. Markopoulou, *J. Chromatogr. B*, 1198 (2022) 123246. https://doi.org/10.1016/j.jchromb.2022.123246.
26. L. G. Hargis, Analytical Chemistry - Principles and Techniques, Prentice-Hall International, London (1988). https://www.scribd.com/document/399558689/101i-pdf
27. M. Q. Al-Abachi, T. Al-Ghabsha and E. Salih, *Microchem. J.*, 41 (1990) 64. https://doi.org/10.1016/0026-265X(90)90096-N.
28. Z. Z. Al-Abdali, Development of spectrophotometric, fluorometric and high performance liquid chromatographic methods for determination of some drug compounds, Ph.D. Thesis, University of Mosul, Mosul, Iraq (2013). https://iqdr.iq/index?lang=en
29. P. Ravisankar, C. N. Navya, D. Pravallika and D. N. Sri, *IOSR J. Pharm.*, 5 (2015) 7. Available: https://www.researchgate.net/publication/338166852_A_Review_on_Step-by-Step_Analytical_Method_Validation