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Determination of the Antiprotozoal and Antibacterial Drug Metronidazole in Blood and Dosage Forms Using a Simple Spectrophotometric Method

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Abstract

A new, precise, and sensitive method for the determination of Metronidazole has been created. Metronidazole has been determined in both blood and dosage forms. The reaction is based on the reduction of the nitro group of metronidazole followed by coupling with the diazotized p-aminodiphenylamine to produce a soluble, colored complex measured at 515 nm. Beer-Lambert law is obeyed over the concentration range from 20 to 100 µg/mL, the molar absorptivity is 1397.88 L/mol.cm.The method has been used for the determination of metronidazole in the blood of the volunteer after 4, 6, 8, and 12 h from the administration of 500 mg tablet showing that the higher level of the drug was found after 6 h from swallowing the drug orally with excellent precision range (RSD% 0.0122-0.0387). The method has been also applied for the estimation of drug content in injection and tablet dosage forms with accuracy ranging from -4.1- to +4.0.

Keyword: Metronidazole, p-aminodiphenylamine, Blood, Dosage forms

Introduction

Metronidazole (MNDZ) it is an efficient antiprotozoal and antibacterial agent used to treat trichomoniasis, amoebiasis, and giardiasis [1]. It decreases the cytokines levels produced during the treatment of COVID-19 infection [2,3]. MNDZ is usually modified in the liver by oxidation of the side-chain to form hydrophilic products, the main two oxidative metabolites of metronidazole are hydroxy and acetic acid derivatives [4,5]. MNDZ binds to the deoxyribonucleic acid of the anaerobic bacteria and protozoa and blocks its replication [6,7]. Chemically MNDZ (Fig. 1) is 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethan-1-ol)with chemical formula $C_6H_9N_3O_3$ and molecular weight 171.16 g/mole) [8]. MNDZ is known as flagyl; metizol; metro; metrogelv and other brand names [9]. The ultraviolet spectrum of MNDZ in aqueous acid exhibits a peak at 277 nm and in aqueous alkali exhibits a peak at 319 nm [9,10].

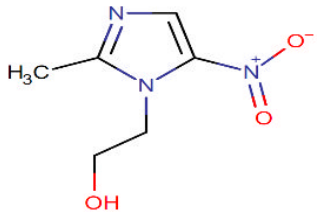


Figure 1. **Metronidazole (MNDZ)**

MNDZ reacts with a silver(I)to form the coordination complex [Ag $(MNDZ)_2NO_3$] and [(Ag $(MNDZ)_2)_2$]SO_4, which exhibits significant antibacterial activity [11].

MNDZ has been estimated in pharmaceutical dosage forms by hyphenated techniques LC-MS/MS chromatographic methods [12-14], electrochemical methods [15-18], and chromatographic techniques [19, 20].

MNDZ has been estimated in pharmaceutical formulations by two flow injection methods using metol (N-methyl-p-aminophenol sulfate) as electron acceptor and the reduced form of metronidazole as an electron donor by two lines manifold procedure in the presence of $NaIO_4$ in the first method and by reverse flow injection manifold in the second [21]. MNDZ has been determined by spectrophotometric methods which are based on the reduction of MNDZ with zinc/HCl, followed by the formation of Schiff base with p-dimethylaminobenzaldehyde [22] and with vanillin [23]. Other spectrophotometric procedures are based on the reduction of the nitro group in MNDZ followed by oxidation with alkaline potassium permanganate [24], also reduction of the nitro group of MNDZ, followed by diazotization of it, and coupling with β-naphthol [25], N-(1-naphthyl) ethylenediamine [26], para-hydroxyl benzaldehyde [27], and with α-naphthol in another literature [28]. MNDZ reacts with chloranilic acid according to the charge-transfer principle, the reaction is taken place in acetone-cetylpyridinium chloride and the formed MNDZ complex was measured at 513 nm. [29], reduced MNDZ reacts with *p*-benzoquinone to form a purple color complex in a methanolic medium measured at 526 nm [30]. spectral changes of metronidazole upon changing the pH of the medium have been followed at 326 nm. The calibration curve has been expressed by the difference in absorbances (ΔA) against concentration [31]. Some techniques for determination of MNDZ are complected and require elaborate, expensive, and may not be available. MNDZ in all spectrophotometric methods acted as a diazotized compound, in this article the reduced MNDZ acts as a coupling agent, and the proposed method exhibits good applicability for the determination of MNDZ in blood as well as in dosage forms.

Material and Methods

Instruments

Absorption spectra were measured on a double beam Jasco V- 630spectrophotometer with 1.0 cm matched glass cells.

Chemicals

All chemicals used were of analytical grade:

Prepared solution

Metronidazole (100 µg/mL), Sodium Nitrite ($NaNO_2$) (1%), Sulphamic acid (3%), hydrochloric acid (1 M), Sodium hydroxide (4 M), P-amino diphenylamine ($1x10^{-3}$ M): 0.0184 g of pure reagent has been dissolved in 10 mL of ethanol followed by dilution to100 mL.

Metronidazole tablet/200 mg (Ajanta Pharma Limited Indian): The content of five tablets has been mixed, pulverized, and weighed. The mean weight of one tablet was 0.3286 g 0.08215 g (equivalent to 0.05 g active component *Metronidazole*) was reduced according to the reduction procedure, then diluted to prepare 100 µg/mL.

Metronidazole tablet/500 mg (Microlab Limited): The content of five tablets has been mixed, pulverized, and weighed. The mean weight of one tablet was 0.65402 g. So, 0.065402 g of tablet powder is equivalent to 0.05 g active component *Metronidazole*) was reduced according to the reduction procedure, then diluted to prepare 100 µg/mL.

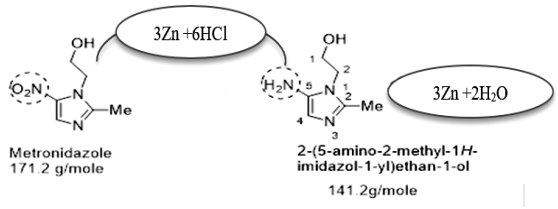
Metronidazole Intravenous Injection 500 mg/100 mL (Pioneer company Iraq-Sulaymaniyah): 10 mL of the Intravenous Injection liquid was reduced according to the reduction procedure, then diluted to prepare 100 µg/mL of the drug sample.

Extraction of MNDZ from human blood

2 mL of human blood samples (of healthy voluntaries) have been collected after 4, 6, 8, and 12 h after oral administration of a single dose 500 mg tablet MNDZ tablet/500 mg (Microlab limited)in heparinized tubes during non-alternative days, 0.5 mL of sodium citrate kit has been added, separation was carried out by centrifugation 5000 periods per second at room temperature, the decantated supernatant was reduced by 0.4 g zinc powder in acidic medium (5 mL of concentrated HCl), mixed with 10 mL of hot distilled water and boiled for 5 min, then cooled in ice, filtrate and finally diluted to make 25 mL with distilled water [33,34].

Reduction step using zinc in acidic medium

This step involves the addition of 0.4 g zinc powder to 0.05 g MNDZ pure powder (provided by the state company of drugs industry and medical appliances), followed by the addition of 5 mL of concentrated HCl, then 10 mL of hot distilled water, after cooling period, the solution was diluted to make 100 mL final volume and 500 µg/mL final concentration. The final solution was diluted to prepare a 100 µg/mL MNDZ working solution. The reaction was expressed by the chemical equation as in scheme 1 [26].



Scheme 1. **Reduction reaction of MNDZ**

Starting conditions of the reaction

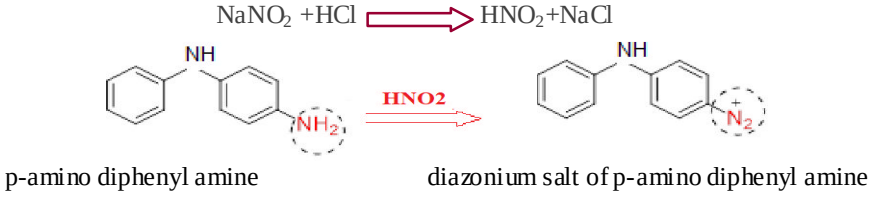
1 mL of sodium nitrite and 0.5 mL of HCl were added to 2 mL of the reagent p-aminodiphenyl amine, followed by the addition of 1.5 mL sulphamic acid after two min, another two min then 1 mL of the reduced metronidazole is added, finally 2 mL sodium hydroxide has been added and the solution is diluted to make 10 mL in a volumetric flask. The formed color was orangish-red measured at 515 nm to give a 0.1679 absorbance value against a blank solution. The effluence of many types and amounts of chemicals on the reaction efficiency indicated by absorbance values have been studied and explained below:

Results and Discussion

Optimization of the Reaction Conditions

The influence of sodium nitrite

The reagent p-aminodiphenyl amine is a primary aromatic amine that can be easily diazotized by the addition of sodium nitrite in an acidic medium to form nitrous oxide. Nitrous oxide reacts with p-aminodiphenyl amine to form diazonium salt pulling a water molecule according to the equations in scheme 2:



Scheme 2. **Formation reaction of diazonium salt**

Practically 0.2, 0.4, 0.8, 1, 1.5, and 2 mL of sodium nitrite (1%) have been added in an acidic solution to the organic reagent ($1x10^{-3}$ M). Fig. 2 shows a maximum at 1.5 mL of sodium nitrite.

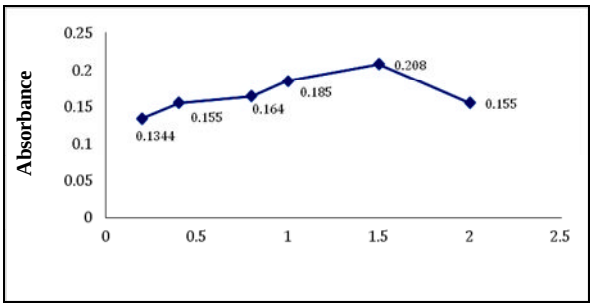


Figure 2. **The influence of the quantity of sodium nitrite**

The influence of acids

The presence of many acids (H_3PO_4, HNO_3, H_2SO_4, HCl, CH_3COOH) with many quantities on the sensitivity of the colored product exhibits a maximum at 0.5 mL of 1 M HCl, while both nitric acid and phosphoric acids exhibit negative effect Fig. 3 summarize the results.

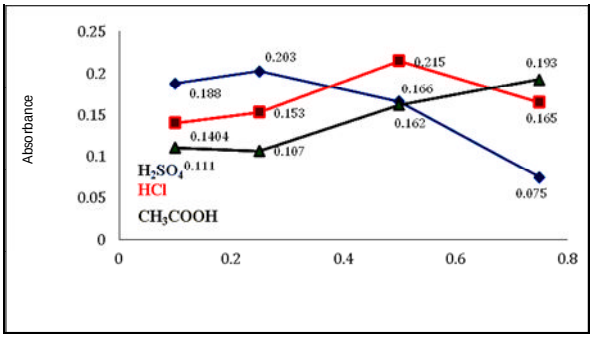


Figure 3. **The influence of the acids**

Adjustment the amount of sulphamic acid

An excess of nitrous oxide may cause an undesirable further reaction, so, sulphamic acid is used to adjust the amount of nitrous oxide according to the below reaction equation:

$$HNO_2 + NH_2SO_3H \longrightarrow N_2\uparrow + H_2O + H_2SO_4$$

Fig. 4 shows that 1.5 mL of 3% sulphamic acid is a convenient amount to remove the excess amount of nitrous oxide.

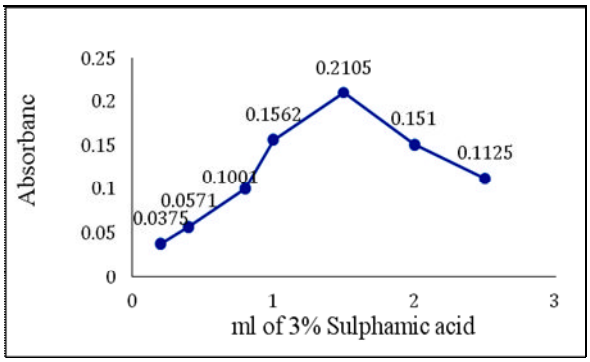


Figure 4. **The influence of the quantity of sulphamic acid**

Select the type and adjustment the amount of base

The diazonium formation requires an acidic medium while the coupling step requires a basic medium to enhance and increase chromophore area. 0.5 mL of 4 M of bases NaOH, KOH, and Na_2CO_3 as basic salts are used for the last requirements. Fig. 5A showed that NaOH is the best choice. Fig. 5B showed that exactly one milliliter is preferred.

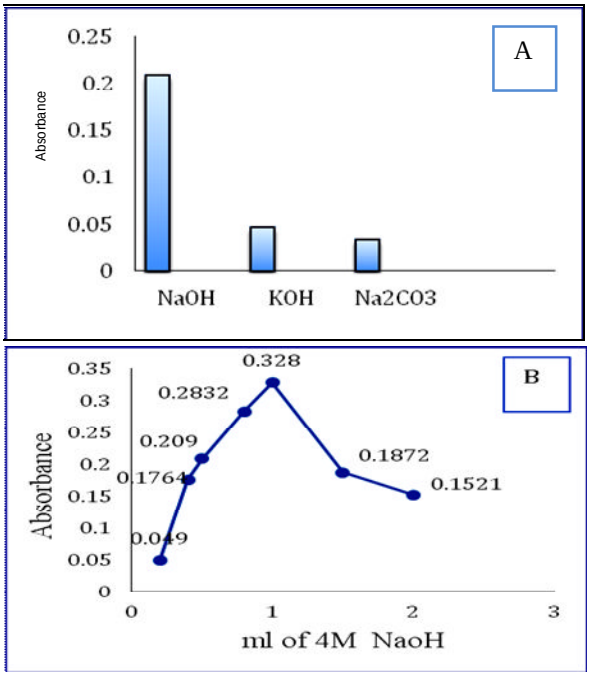


Figure 5. **A: Selection of base B: Selection of NaOH amount**

Adjustment the amount of organic reagent agent

1.5, 2, and 2.5 mL of p-aminodiphenyl amine agent ($1x10^{-3}$ M) against 10, 20, 30, 40, 50, 75, 100, and 125 µg of MNDZ in final volume 10 mL under the above-selected conditions have been followed. Table 1 indicates that 1.5 mL of the reagent produces a higher correlation and it is selected.

***Table 1*. Influence of p-aminodiphenyl amine on the absorbance of increasing concentrations of MNDZ.**

Volume of p-aminodiphenyl amine (mL)	Absorbance/µg of MNDZ						R^2
	10	30	50	75	100	125	
1.5	0.011	0.0503	0.325	0.3801	0.6110	0.953	0.974
2	0.022	0.0460	0.3218	0.3620	0.661	1.0532	0.952
2.5	0.039	0.073	0.1965	0.335	0.629	1.066	0.928

Effect of standing time

Table 2 explains the effect of standing time after two steps:

***Table 2*. Effect of standing time**

Time (min.)	immediately	1	2	3	5	7
After reaction of reagent with nitrous oxide	0.2447	0.2547	0.3280	0.3210	0.3200	0.3100
After release the excess of nitrous oxide	-----	0.321	0.3341	0.3400	0.335	0.330

1- After the diazonium step (addition of sodium nitrite), in which the measurements have been taken after 0, 1, 2, 3, 5, and 7 min of the addition of sodium nitrite. From Table, 2 min is sufficient.

2- After the release the excess of nitrous oxide step (addition of sulphamic acid), in which the same above periods have been followed as shown in Table 2, 3 min is sufficient.

Effect of surfactant and sequence of addition

As Table 3 show, the cationic surfactant cetylpyridiniumchloride (CPC) relatively enhance the value of absorbance when 2 mL of the surfactant is added, while Fig. 5A exhibits that 2 mL of CPC produces the maximum enhancements following sequence 4 as explained in Fig. 5A.

***Table 3*. Influence of surfactant.**

Surfactant solution (1×10^{-3} M)	Absorbance/ order of addition*
Sodium dodecyl sulphate (SDS)	0.328
Cetyltrimethylammonium bromide (CTAB)	0.319
Cetylpyridinium chloride (CPC) 0.1%	0.345

* p-aminodiphenyl amine (PADPA) + surfactant (S) + Hydrochloric acid (H)+sodium nitrite (N) + Sulphamic acid (F) + Metrazol (D) + sodium hydroxide

The effect of different volumes of CPC 0.1% has been studied, two mL exhibits the best results as shown in Fig. 6A and six sequences of additions have been checked, as shown in Fig. 6B, sequence 4 is the best one and it is followed by pre- and post-experiments.

Sequence 1: p-aminodiphenyl amine (PADPA) + Hydrochloric acid (H)+ surfactant (S) +sodium nitrite (N) + Sulphamic acid (F) + Metrazol (D) + sodium hydroxide

Sequence 2: PADPA + H + N+ F + S + D + B

Sequence 3: PADPA + H + N + S + F + D + B

Sequence 4: PADPA + H + N + F + D + B + S

Sequence 5: PADPA + H + N + F + D + S + B

Sequence 6: PADPA + N + H + F + D + B + S

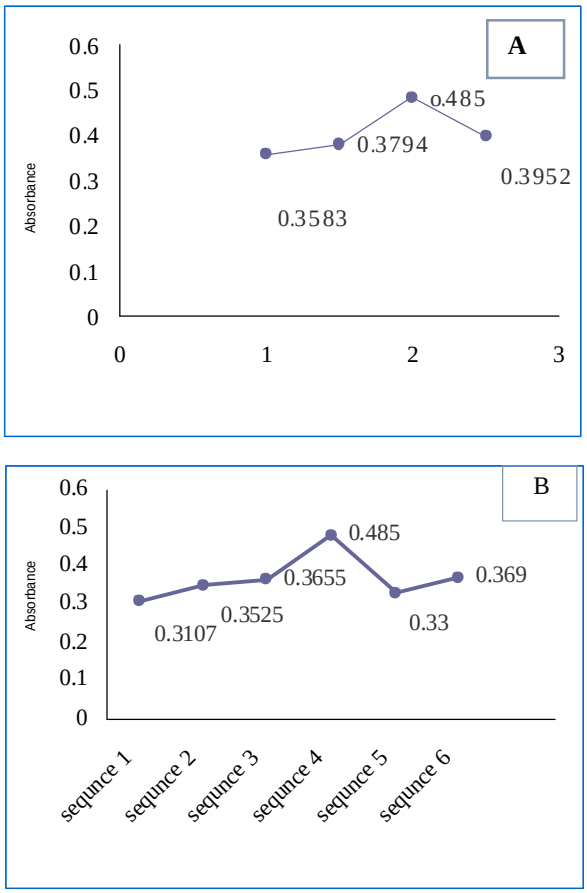


Figure 6. **A: Effect of CPC B: Effect of the sequence* of addition in the presence of CPC**

Absorption spectrum and calibration curve

Under the developed reaction procedure, the absorption spectrum has been taken, and the amount and sequence of additions are as follows:

1.5 mL of the reagent, 0.5 mL of 1 M HCl, 1.5 mL of sodium nitrite, standing for 2 min, 1.5 mL of sulphamic acid, and another 3 min to release the excess of nitrous oxide, 5 mL of MNDZ (100 µg/mL), 1 mL of 4 M of bases NaOH, finally 0.2 mL of 1% M CPC, dilution to complete 10 mL in a volumetric flask. A blank solution has been prepared in the same way but in the absence of MNDZ. From Fig. 7, the maximum absorbance of the formed complex at 515 nm is about 0.4855.

Between 10-120 µg/mL of MNDZ solution has been measured following the same above conditions for estimation of a calibration curve, Fig. 8 shows the linearity is between 20 to 100 µg/mL of MNDZ.

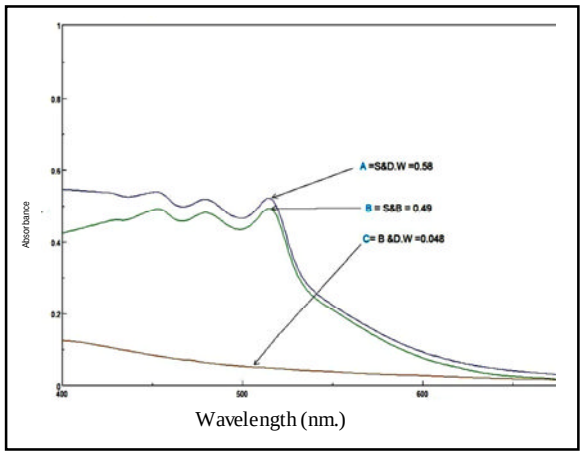


Figure 7. **The absorption spectrum of 50 ppm of A: Sample against distilled water B: Sample against blank and C. Blank against distilled water**

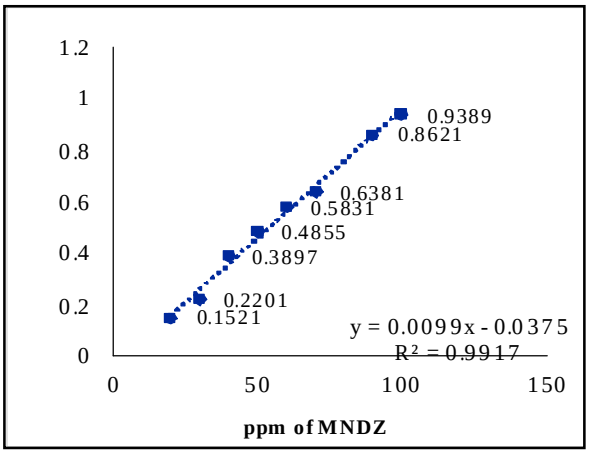


Figure 8. **The calibration curve of MNDZ**

Accuracy and precision of the calibration curve

The accuracy and precision of the calibration curve have been estimated by making measurements of three different concentrations with many replications as mentioned in Table 4.

Table 4. **Accuracy and precision of the calibration curve.**

MNDZ taken (ppm)	Recovery %	The mean of recovery %*	Relative standard deviation %*	The mean of relative standard deviation %*
30	98.5		0.4620	
50	100	99.46	0.5118	1.0559
90	99.9		2.194	

* Average of five determination

The calculated molar absorptivity is 1397.88 L/mole.cm, LOD is 0.358 µg/mL, LOQ is 1.956 µg/mL, while the mean of recovery is 99.46 %, and of relative standard deviation is µg/mL.

Nature of the formed complex

A brief study on the nature of the complex using the job's and mole ratio method exhibits a one-to-one reaction ratio between the diazotized p-aminodiphenylamine and the coupling agent (the reduced MNDZ). (Fig. 9 A asnd B).

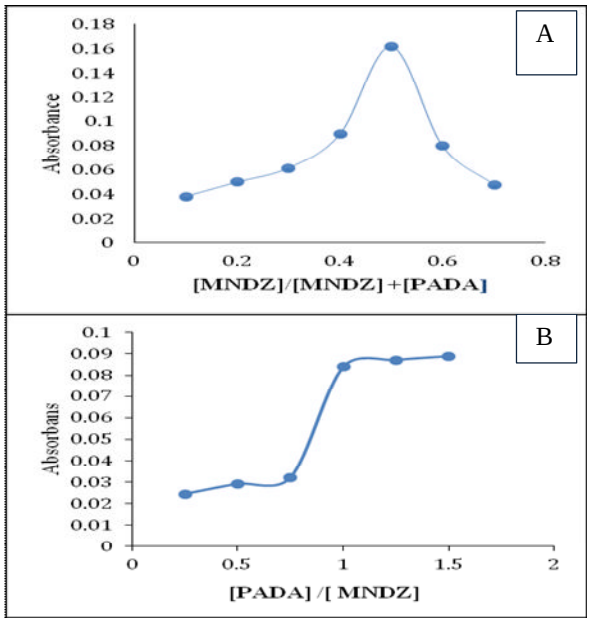
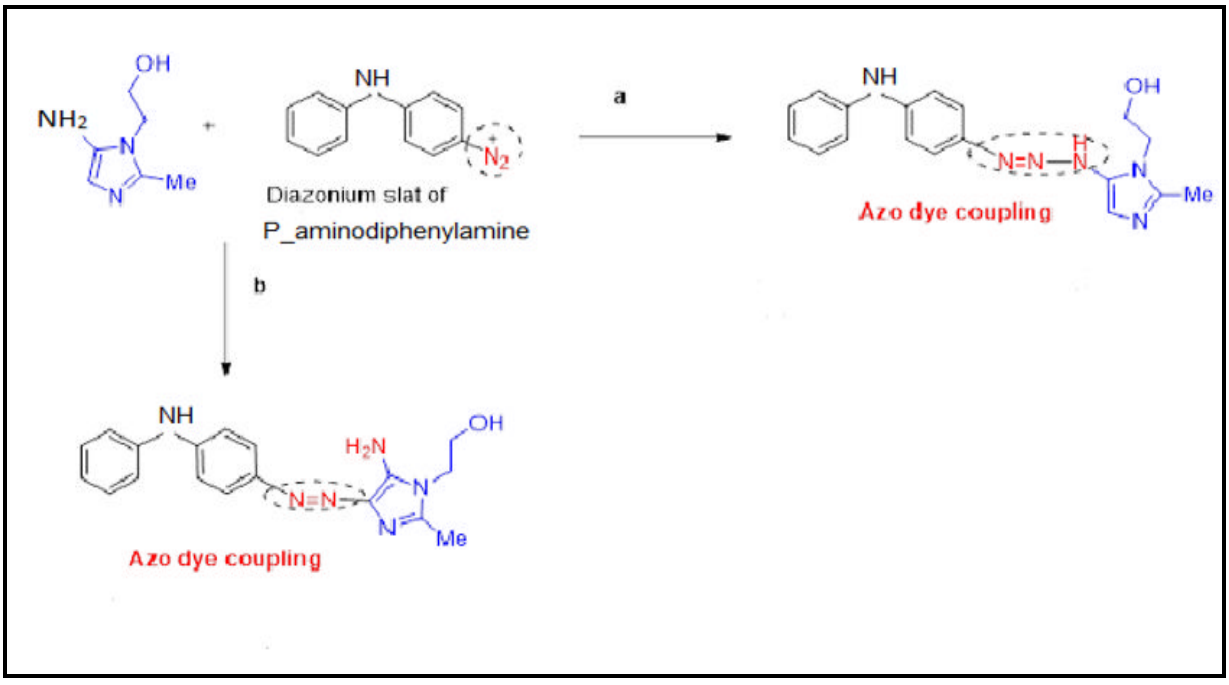


Figure 9. **A: Job' s method and B: mole ratio method to confirm the nature of the complex**

As the obtained ratio was 1:1, the expected structure may be occurred either according to a suggestion or b suggested structure as in scheme 3 below:



Scheme 3. **The suggested structure of the colored azo product**

The stability constant (Ks) of the colored azo product

Table 5 exhibits good stability of the complex, the average conditional stability constant is 1.14 x10^5 L/mole.

***Table 5*. stability of the azo colored complex.**

mL of ($1x10^{-3}$) MNDZ	As*	Am**	α***	Ks (1/ mol)	Mean of Ks (1/ mol)
0.5	0.0219	0.0301	0.272	$1.97x10^5$	
1	0.0401	0.0650	0.383	$0.42x 10^5$	$1.14 x10^5$
1.5	0.0692	0.0891	0.233	$1.03x 10^5$	

*Absorbance of the same amount of sample and reagent (1 sample:1 reagent)
**Absorbance of a maximum amount of reagent (1 sample:10 reagent)
*** Ratio of dissociation (α= Am-As/As)

Real sample analysis

Determination of MNDZ in human blood

MNDZ is metabolite mainly to 2–hydroxymethylmetronidazole and 2–methyl–5–nitroimidazol–1–acetic acid, distributed quickly, and excreted mainly as glucuronic acid derivative in the urine during 48 hours with less than 10% of the dose as excreted as unchanged MNDZ [13,16].

1, and 1.2 mL of extracted MNDZ from human blood samples have been taken, treated according to the recommended procedure, diluted to make 10 mL, and measured at 515 nm. the results are listed in Table 6 and summarized in Fig. 10.

***Table 6*. Determination of MNDZ in human blood -many hours after administration.**

After administrated period (hours)	mL of extracted blood sample	xi	$\bar{x}$	Concentration of MNDZ found (ppm)	(xi-$\bar{x}$)	$(xi-\bar{x})^2$	R.S.D %
4	1	0.2090	0.2088	21.09	$1 x 10^{-4}$	$1 x 10^{-8}$	0.0122
		0.2089			$1 x 10^{-4}$	$1x 10^{-8}$	
		0.2087			$-1 x 10^{-4}$	$1 x 10^{-8}$	
	1.2	0.2183	0.2184	22.06	$1 x 10^{-4}$	$1 x 10^{-8}$	0.0173
		0.2183			$-1x 10^{-4}$	$1 x 10^{-8}$	
		0.2186			$2 x 10^{-4}$	$4 x 10^{-8}$	
6	1	0.2195	0.2193	22.15	$2 x 10^{-4}$	$4 x 10^{-8}$	0.0331
		0.2190			$-3 x 10^{-4}$	$9x 10^{-8}$	
		0.2196			$3 x 10^{-4}$	$9 x 10^{-8}$	
	1.2	0.2401	0.2403	24.27	$-2 x 10^{-4}$	$4 x 10^{-8}$	0.0387
		0.2408			$5x 10^{-4}$	$25 x 10^{-8}$	
		0.2402			$-1 x 10^{-4}$	$1 x 10^{-8}$	
8	1	0.0925	0.0922	9.31	$3 x 10^{-4}$	$9 x 10^{-8}$	0.0308
		0.0919			$-3 x 10^{-4}$	$9 x 10^{-8}$	
		0.0922			$1 x 10^{-4}$	$1 x 10^{-8}$	
	1.2	0.1109	0.1107	11.18	$2 x 10^{-4}$	$4 x 10^{-8}$	0.0173
		0.1108			$1x 10^{-4}$	$1 x 10^{-8}$	
		0.1107			$-1 x 10^{-4}$	$1 x 10^{-8}$	
12	1	0.0248	0.0247	2.49	$1 x 10^{-4}$	$1 x 10^{-8}$	0.0212
		0.0249			$2 x 10^{-4}$	$4 x 10^{-8}$	
		0.0245			$-2 x 10^{-4}$	$4 x 10^{-8}$	
	1.2	0.0406	0.0405	4.09	$1 x 10^{-4}$	$1 x 10^{-8}$	0.0308
		0.0402			$-3 x 10^{-4}$	$9 x 10^{-8}$	
		0.0408			$3 x 10^{-4}$	$9 x 10^{-8}$	

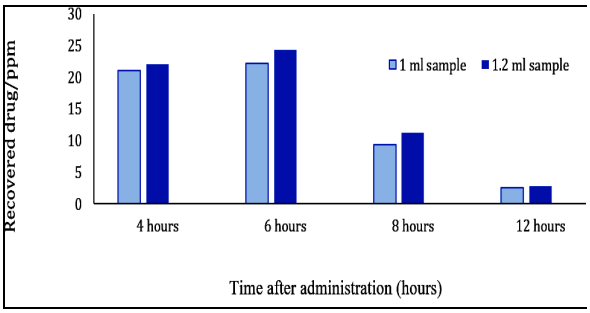


Figure 10. **MNDZ content in human blood after many hours after administration**

Determination of MNDZ in pharmaceutical preparations

The suggested method has been applied for the determination of MNDZ in different dosage forms. Table 7 shows wide application ranges with excellent recovery values.

Table 7. **Determination of MNDZ in pharmaceutical preparations.**

Pharmac-eutical preparations of MNDZ.	MNDZ taken (µg/mL)	Absorb-ance of standard	Absor-bance of sample	Recovery * %	Error %
Metronidazole injection 500 mg / pioneer company	30	0.2201	0.2131	96.8	-3.2
	50	0.4855	0.4682	96.4	-3.6
	90	0.8621	0.8415	97.6	-2.4
	100	0.9389	0.9023	96.1	-3.9
Metronidazole tablet 500 mg /microlab limited	30	0.2201	0.2291	104	+4.0
	50	0.4855	0.4845	99.7	-0.3
	80	0.8012	0.8034	100.2	+0.27
	100	0.9389	0.9021	95.9	-4.1
Metronidazole tablet 200 mg / Ajanta pharma limited / India	30	0.2201	0.2251	102.2	+2.27
	50	0.4855	0.4782	98.4	-1.5
	90	0.8621	0.8515	98.7	-1.22
	100	0.9389	0.9123	97.1	-2.83

Comparison of the method with related published methods

A comparison of the proposed method with the other literature methods shows that the present method has a higher range of linearity, lower detection limit, and lower quantitation limit as shown in Table 8.

Table 8. **Comparison of the method with related published methods.**

Parameter	Present Method	Literature method [34]	Literature method [35]
Method	Diazotization of the reagent	Diazotization of the reagent	Diazotization of MZOL
Reagent	p-aminodiphenylamine	p-amino benzophenone	α-naphthol amine
Wavelength (nm)	515	431	510
Beers law range (µg/mL)	20-100	2-24	2-12
Limit of detection (µg/ml)	0.0351	0.2407	0.1142
Limit of quantification (µg/mL)	0.1172	0.8024	0.3805
Molar absorptivity ($L.mol^{-1}.cm^{-1}$)	$0.1379x10^4$	$2.284x10^4$	$1.5x10^4$
Sandell's sensitivity index (µg cm^{-2})	0.1010	0.0074	0.0114
Application	Dosage forms and biological sample	Dosage forms and biological sample	Tablet

Conclusion

Metronidazole has been determined in blood, tablet, and injection using one simple diazotization-coupling reaction. The drug level in blood show maxima after 6 h after swallowing the drug orally with excellent precision range (RSD% 0.0122-0.0387). The estimation of drug content in injection and tablet exhibits high accuracy ranging from -4.1- to +4.0. The technique is simple, the

method is sensitive, and does not consume energy, heat, buffers, or pH adjustment.

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Conflict of Interest

The authors declare no conflict of interest.

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