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Spectrophotometric Determination of Metronidazole in Pharmaceutical Preparations and in Human Blood samples

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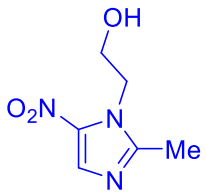
Abstract

This research investigates a sensitive quantitative estimation of metronidazole (MZOL) in each of its pharmaceutical preparations and its level in human blood with one color reaction and one simple spectrophotometric technique. Metronidazole the antibiotic and antiprotozoal medication is determined by the reduction reaction of MZOL to 2-(2-Methyl-5-amino-1H-imidazole-1-yl) ethanol, followed by coupling with diazotized p-amino benzophenone (PABPh) reagent. The produced color complex is measured at 431 nm. The proposed method is successfully applied for the determination of metronidazole in different dosage forms (tablet, suspension, and intravenous injection) with high precision (RSD% from ±0.011 to ±2.3).as well as it applied for the determination of metronidazole in human blood samples of healthy voluntaries easily and accurately after 4, 6, 12 and 24 hour of oral administration with relative standard deviation not more than ±1.922 which provide the ability for therapeutic monitoring follow up of metronidazole in blood.
Keywords: Metronidazole, spectrophotometry, pharmaceutical preparations, blood samples

1. Introduction

Metronidazole (MZOL) (Figure 1)is an antibiotic, derived from nitroimidazole, and it is used in treatment of anaerobic bacterial infections and parasites that infect joints, brain spinal cord, skin, vagina, stomach and liver.[1]



2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethan-1-ol
Chemical Formula: $C_6H_9N_3O_3$
Molecular Weight: 171.16

Figure 1. The empirical formula, molecular weight and the chemical structure of Metronidazole

The physical properties of Metronidazole are white to pale yellow colour powder which may be changed to dark colour when it exposed to light, its melting point is 158-160°C, pKa is 2.5 (dissociation constant), and it soluble in water, ethanol, and slightly soluble in ether [2]. Metronidazole is recently used in regime of COVID19[3] because of its role in decrease level of several cytokines in blood which already increase in COVID19 patients. Also, MZOL could decrease neutrophil-generated ROS (Reactive Oxygen Species) during the inflammation process [3].

There are various methods that used in determination of pharmaceuticals and drugs in human body, these methods are LC-MS/MS chromatographic method [4], electrochemical [5], HPLC and HPTLC [6, 7], polarographic [8], spectrophotometry [9], nano-composite electochemical [10] official methods [11, 12], voltametric [13], volumetric [14], derivative spectrophotometry[15], gas-chromatographic, TLC [16, 17, 18], and flow injection analysis [19-21].

The maximum wavelength of aqueous acid solution of Metronidazole is 277nm and shifts to be 319 nm in aqueous alkali medium, and it completely absorbed after oral administration, distributed in the body, oxidized to 2–hydroxymethylmetronidazole and 2–methyl–5–nitroimidazol–1–acetic acid, and it conjugated with glucuronic acid. About 70 to 80% of a dose is excreted in the urine in 48 h with less than

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10% of the dose as unchanged drug, other ratio is excreted as metabolized form of Metronidazole. A general reaction sequence for the electrochemical degradation of MTZ is proposed in Figure 2 [22, 23].

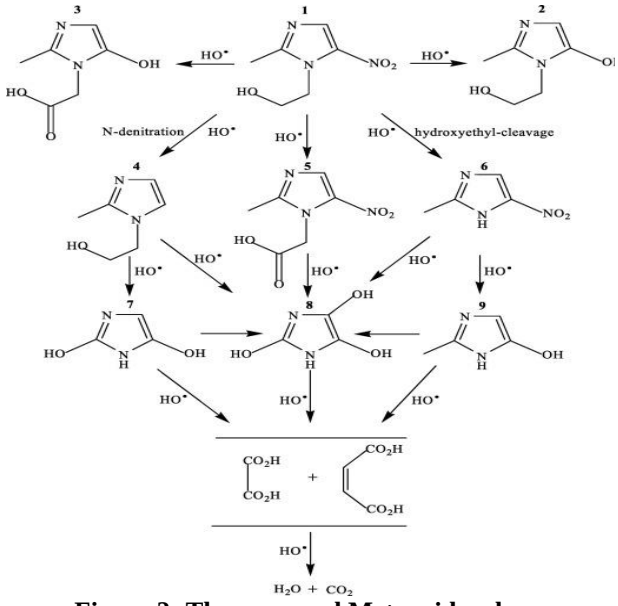


Figure 2: The proposed Metronidazole degradation pathway [22,23]

MZOL and its primary metabolite, 2-hydroxymetronidazole has been followed in human blood by HPLC method using paracetamol as an internal standard. Methanol was used to denature plasma proteins [23,24]. MZOL was also extracted from plasma, prostate gland and seminal vesicles of rat using ethyl acetate followed by determination step using reverse-phase high-performance liquid Chromatography [25].

The aim of this work is determined MZOL in a simple, rapid and sensitive method in both of its pharmaceutical preparations and in blood of healthy voluntaries easily and precisely. The presence of the same functional groups (electron donating group) in MZOL and its metabolites make the work possible by using one procedure and same simple technique.

2. Experimental

a. Apparatus

The following instruments and equipment have been used:

- Absorbances were performed using single beam- SPECTRO UVD-3000/UVD-3200Serial no.8UVD- 11001Labomed.Inc. U.S.A.
- Absorption spectra were performed on double-beam Jasco V- 630spectrophotometer with 1.0 cm matched quartz cells.
- Wisd electrothermal heater and stirrer.
- pH measurements were performed using HANNA 301 pH meter
- BEL balance was used for weight measurements.

b. Reagents

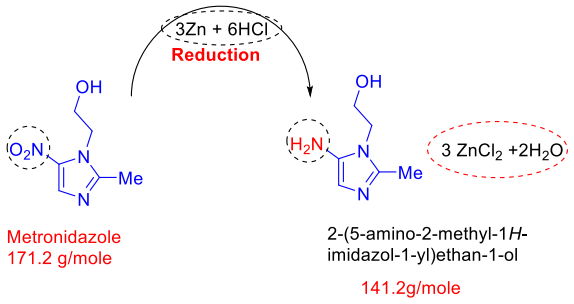
All chemicals used were of analytical grade.

- Reduced Metronidazole (100 µg.ml-1)
- Sodium Nitrite (NaNO2) 1%
- Sulphamic acid (3%)
- Sulphuric acid (1M)
- Sodium hydroxide (1M)
- P-amino benzophenone (1X10-3M): 0.0184g of pure reagent has been dissolved in 10 ml of ethanol followed by dilution to100 ml.
- Metronidazole tablet/250mg (Indian): The content of five tablets has been mixed, pulverized, and weighed. The mean weight of one tablet was 0.6 g.0.024 g (equivalent to 0.01g active component MZOL) was reduced according to the reduction procedure, then diluted to prepare 100 µg/ml.
- Metronidazole tablet/200mg (Indian): The content of five tablets has been mixed, pulverized, and weighed. The mean weight of one tablet was 0.29 g.0.0145 g (equivalent to 0.01g active component MZOL) was reduced according to the reduction procedure, then diluted to prepare 100 µg/ml. prepare 100 µg/ml.
- Metronidazole suspension 200 mg/5ml (Iraq-Erbil):0.5 ml of suspension has been diluted was reduced according to the reduction procedure, then diluted to prepare 200 µg/ml of the drug solution, further dilution to prepare 100 µg/ml drug solution.
- Metronidazole Intravenous Injection500 mg/100ml (Iraq-Sulaymaniyah) :2 ml of the Intravenous Injection liquid was reduced according to the reduction procedure, then diluted to prepare 100 µg/ml of drug sample.

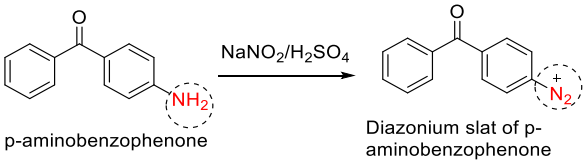
c. Chemical reactions

The determination process of metronidazole undergoes into four chemical reaction steps as explained in scheme 1.

1. Reduction step using zinc in acidic medium

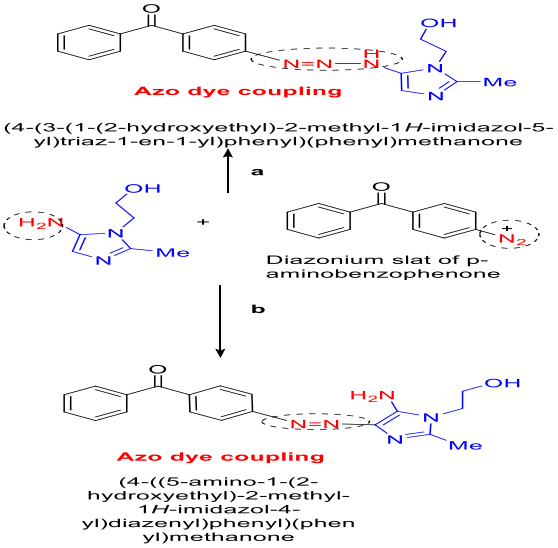


2. Diazotization of organic reagent



3. Remove the exceed amount of nitrous oxide to prevent oxidation of the coupling agent

4. Coupling reaction between the diazonium salt of reagent in alkaline medium to produce the colored azo dye.



Scheme 1: chemical reaction steps

d. Reduction of Metronidazole

The reduction of nitro group on the five position of imidazole ring of Metronidazole to amino group provides a good electrophile for coupling reaction step, therefore, Metronidazole has been reduced to 2-(2-Methyl-5-amino-1H-imidazol-1-yl) ethanol by boiling of 0.05 g Metronidazole powder with 0.4 g of Zn, 5 ml of concentrated HCl, and10-12 ml hot distilled water, the solution is then cooling in ice, filtrated and diluted to 100 ml to produce 500µg/ml, further dilution is required to prepare 100 µg/ml Metronidazole solution.

3. Results and Discussion

Study of the optimum reaction conditions

Selection of Organic reagent

Two organic reagents have been cheeked as diazotized compound, p-aminobiphenyl amine and p-amino benzophenone using 3ml of 1% of each reagent, diazotized by 1.5 ml of sodium nitrite in acidic solution (2ml of HCl), and coupled with 3ml of the reduced metronidazole (100 µ/ml) in basic medium, the results listed in Table 1. Show that p-amino benzophenone produces a complex at higher wavelength.

Table 1: Selection of suitable reagent

Reagent	Absorbance	λ_{max}
p-aminodiphenyl amine	0.137	400
p- aminobenzophenone	0.129	420

Selection of acid and its amount

Diazonium reaction require acidic medium to liberate nitrous oxide simultaneously, the effect of different amount of many acids on absorption intensity has been studied and the results have been listed in Table 2 which indicates that 0.5 ml of H_2SO_4 is the best choice in which it gives the higher absorbance value.

Effect of nitrite amount with time

Between 0.1 ml to 1.5 ml of NaNO2 (1%) has been checked with a standing time from 0 to 6 min, the results are listed in (Table 3).

Table 3 indicate that the reaction exhibits maximum absorbance when 1 ml of sodium nitrite is added with one minute as standing time.

Effect of sulphamic acid amount with time

Between 0.3-1.5 ml of 3% of sulphamic acid solution was added, and absorbance of the solutions was followed at different standing time. Table 4 show that one and half millilitre of sulphamic acid left for five minutes is sufficient to destroy the excess of sodium nitrite.

From Table 4 the reaction exhibits maximum absorbance when 1.5 ml of sulphamic acid is added with five minutes as standing time.

Effect of coupling agent

Diazonium salts are excellent electrophiles that are directly through nitrogen coupling, or releasing a dinitrogen molecule to form in situ aryl carbocation species [13]. The correlation of increasing amount of the diazotized p-amino benzophenone (PABPh) with increasing volumes of the reduced metronidazole has been cheeked by follow the correlation coefficient of linear curves produced. Table 5 shows that the best correlation is produced by using one millilitre of the diazotized reagent, therefore, it has been used in subsequent analytical steps while 3ml was used in the above steps.

Selection of base and its amount

The alkalinity of medium of the coupling step enhance the resonance and increase the area of the chromophore, therefore, four types of bases or basic salts at different volumes (1-5) ml of one molar of each have been tested for their effect on the absorption intensity of the dye formed. The results

are listed in (Table 6) and exhibits that 3.5 ml of NaOH is the best choice.

Stability of reaction

The Stability of the colored product against time has been followed after complete the reaction components at different period of time begin with zero end by 60 minutes. The results are listed in Table 7, and shows that the color is developed immediately and stay stable for 10 min which is sufficient period for make measurements.

Table 2: Selection of acid and its amount

Acid solution used (1M)	Variable*	Absorbance/ml of acid 0.5	1	1.5	2	2.5	3
HCl	S	0.210	0.166	0.127	0.121	0.087	0.077
	B	0.084	0.076	0.074	0.046	0.024	0.020
H_2SO_4	S	0.279	0.267	0.185	0.183	0.092	0.068
	B	0.044	0.076	0.100	0.096	0.023	0.016
HNO3	S	0.231	0.224	0.189	0.068	0.063	0.05
	B	0.046	0.044	0.043	0.009	0.012	0.017
H_3PO_4	S	0.242	0.221	0.210	0.126	0.092	0.045
	B	0.115	0.113	0.025	0.020	0.015	0.012
CH_3COOH	S	0.133	0.113	0.110	0.061	0.056	0.063
	B	0.071	0.103	0.072	0.027	0.020	0.032

* S:sample , B:blank

Table 3: Effect of sodium nitrite amount with time

Ml of $NaNO_2$ solution used (1%)	Variable	Absorbance/ time (min.) 0	1	2	3	4	5	6
0.1	S	0.238	0.239	0.257	0.250	0.231	0.179	0.132
	B	0.059	0.039	0.050	0.029	0.061	0.064	0.051
0.3	S	0.224	0.235	0.204	0.221	0.191	0.178	0.129
	B	0.065	0.049	0.072	0.042	0.055	0.050	0.091
0.5	S	0.208	0.236	0.213	0.232	0.180	0.162	0.102
	B	0.062	0.029	0.024	0.039	0.072	0.045	0.022
0.7	S	0.233	0.241	0.238	0.255	0.197	0.150	0.113
	B	0.047	0.036	0.040	0.037	0.041	0.039	0.047
1	S	0.231	0.354	0.289	0.269	0.188	0.150	0.177
	B	0.036	0.041	0.021	0.011	0.026	0.006	0.018
1.2	S	0.202	0.221	0.273	0.249	0.172	0.144	0.111
	B	0.045	0.043	0.027	0.032	0.025	0.022	0.017
1.5	S	0.242	0.236	0.253	0.268	0.240	0.176	0.144
	B	0.013	0.093	0.072	0.044	0.061	0.054	0.039

Table 4 :Effect of sulphamic acid amount with time

ml of Sulphamic acid solution (3%)	Variable	Absorbance / minute standing time 1	2	3	4	5	6
0.3	S	0.032	0.209	0.114	0.167	0.210	0.191
	B	0.030	0.023	0.004	0.020	0.019	0.016
0.5	S	0.092	0.120	0.187	0.159	0.267	0.230
	B	0.042	0.016	0.002	0.015	0.025	0.032
1	S	0.346	0.251	0.186	0.215	0.189	0.172
	B	0.046	0.031	0.023	0.020	0.027	0.024
1.5	S	0.281	0.237	0.208	0.281	0.416	0.257
	B	0.037	0.025	0.012	0.036	0.025	0.030

Table 5 :Effect of coupling agent

ml of PABPh	Variable	Absorbance / µg of MZOL 4	8	12	16	20	24	r^2
1	S	0.181	0.192	0.245	0.267	0. 298	0.311	0.9661
	B	0.016	0.021	0.061	0.033	0.081	0.046	
2	S	0.172	0.231	0.266	0.278	0.289	0.331	0.9277
	B	0.042	0.032	0.018	0.024	0.031	0.076	
3	S	0.169	0.252	0.395	0.411	0.430	0.441	0.8300
	B	0.032	0.038	0.038	0.031	0.029	0.066	
4	S	0.162	0.247	0.365	0.381	0.392	0.420	0.8515
	B	0.022	0.027	0.023	0.068	0.062	0.044	
5	S	0.180	0.228	0.343	0.362	0.371	0.400	0.8746
	B	0.011	0.031	0.020	0.015	0.060	0.063	
6	S	0.176	0.187	0.298	0.336	0.342	0.381	0.9087
	B	0.076	0.032	0.041	0.019	0.028	0.030	

Table 6 :Selection of base and its amount

Base used(1M)	variable	Absorbance/ml of base used 1	1.5	2	2.5	3	3.5	4	4.5	5
NaOH	S	0.211	0.238	0.249	0.264	0.272	0.368	0.301	0.302	0.275
	B	0.031	0.104	0.093	0.040	0.035	0.008	-0.031	-0.004	-0.003
Na_2CO_3	S	0.179	0.159	0.184	0.186	0.187	0.200	0.233	0.223	0.232
	B	0.141	0.128	0.081	0.069	0.070	0.052	0.002	0.017	0.001
NaH CO_3	S	0.119	0.169	0.173	0.176	0.184	0.198	0.192	0.189	0.184
	B	0.001	0.005	0.001	0.006	0.003	0.004	0.012	0.014	0.090
KOH	S	0.197	0.275	0.295	0.294	0.299	0.300	0.294	0.287	0.278
	B	0.049	0.087	0.047	0.011	0.010	0.004	0.002	0.000	0.000

Table 7 :Stability of the colored product

Time min	0	5	10	15	20	25	30
Absorbance 12 µg ml^{-1}metronidazole	0.254	0.248	0.240	0.235	0.229	0.221	0.215
	35	40	45	50	55	60	
	0.221	0.221	0.220	0.194	0.188	0.180	

Absorption spectrum

The figure below has been taken for determination of 3ml of 100 µg/ml of metronidazole under the optimum reaction conditions, and it exhibits 431 nm as the wavelength of maximum absorbance of the formed azo-dye.

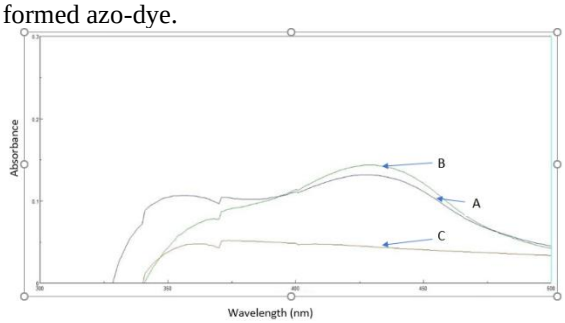


Figure 2. The absorption spectrum of A: MZOL azo dye against blank B: MZOL azo dye against distilled water C: blank against distilled water

Recommended procedure and calibration curve

To series of 25 ml volumetric flasks,1ml of 1 % p-amino benzophenone, 1 ml of 1% $NaNO_2$, 0.5 ml of H_2SO_4 (1M), (gentle shaking for 1 min.), 1.5 ml of 3% sulphamic acid (standing for 5 min.), increasing volumes (0.1-7) ml of 100 µg.ml^{-1} reduced drug solution and finally 3.5 ml of NaOH (1M) have been added, the volumes were completed with distilled water, the absorbances that is related to the real present amount of MZOL have been measured at 431 nm against blank solution. Fig.3.

From the standard curve Beers law range is from 2 to 24 µg/ml, the calculated molar absorptivity is $2.284x10^4$ l. mol-1.cm^{-1}, Sandell's sensitivity index is $7.4956\ x10^{3-}$ µg.cm^{2-}, the correlation coefficient is 0.9964,the linearity of the absorbances-concentration values are from 2 to 24 µg/ml, LOD equal to 0.2407 and LOQ equal to 0. 8024.The accuracy of the standard curve is from +0.04 to+1.14, while the precision is from ± 0.1647 to ±0.887 calculated of three different concentrations and replicate five times as shown in Table 8.

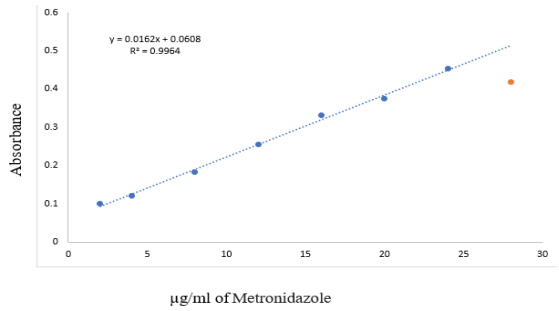


Fig.3 Standard curve for estimation of MZOL

Application of the method

The proposed method has been applied for determination of MZOL in tablet/250mg, tablet/ 200mg (Indian), Suspension 200mg/5ml (Iraq-Erbil), and Intravenous Injection500 mg/100ml (Iraq-Sulaymaniyah). Table (9) show the good accuracy (ER% from -1.6 to +1.6) and high precision (RSD% from ±0.011 to ±2.3).

Standard addition method

The proposed method has been validated by standard addition method applied to determine (Metronidazole tablet/250mg (Indian**).** Into two series 10-ml of volumetric flasks 0 to 3.5 ml of the MZOL standard solution (100 µg.ml^{-1}) was added to the two series, then 0.5 ml of the dosage form solution was added to the first series and 1.0 ml to second series of flasks, followed by the optimum amount at the same order of addition as in the recommended procedure. The absorbance was measured at 431 nm. The results in Fig 4, Fig 5, and Table 11 exhibits the satisfactory results of the proposed method.

Determination in biological samples

3 ml of human blood samples (of healthy voluntaries) have been collected after 4,6,12, and 24 hours after oral administration of Metronidazole 250mg tablet (Indian) in heparinized tubes during different days,0.5 ml of sodium citrate has been added, separation was carried out by centrifugation 5000 period per second at room temperature, the decantated supernatant was reduced by 0.4 gm zinc powder in acidic medium (5 ml of concentrated HCl),mixed with 10-12 ml of hot distilled water and boiled for 5 minutes, then cooled in ice for 15 min., followed by filtration and finally diluted to make 50 ml with distilled water. 2,4, and 6 ml of prepared samples has been taken, treated according the recommended procedure, and diluted to make 25 ml, the results are listed in table 10 and summarized as in figure 6.

Table 12 show an excellent relative standard deviation (RSD is from ±0.228 to ±1.922 %) for the determination of MZOL in blood at different period of time

Table 8: accuracy and precision of the proposed method

Amount of MZOL taken µg/ml	Recovery%*	Relative error%*	Relative standard deviation RSD%*
4	101.14	+1.14	±0.887
12	100.93	+0.93	±0.1647
24	100.04	+0.04	±0.286

*Average of five determinations.

Table 9: Application of the proposed method to pharmaceutical preparations of MZOL

Pharmaceutical preparations	µg/ml	Recovery%	RE%*	RSD%*
Metronidazole tablet/250mg (Indian)	4	101.6	+1.6	0.8
	12	98.4	-1.6	0.619
	24	100.4	+0.4	0.252
Metronidazole tablet/200mg (Indian)	4	99.1	-0.9	0.704
	12	100.7	+0.7	0.011
	24	99.7	-0.3	0.270
Metronidazole Suspention 200mg/5ml (Iraq-Erbil)	4	98.5	-1.5	1.006
	12	99.6	-0.4	2.340
	24	99.3	-0.7	0.719
Metronidazole Intravenous Injection500 mg/100ml (Iraq-Sulaymaniyah)	4	100.8	+0.8	1.935
	12	100.3	+0.3	1.169
	24	100.2	+0.2	0.570

*Average of five determinations

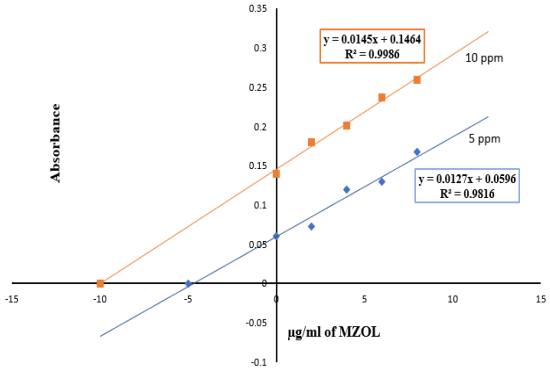


Fig.4 Standard addition method of Metronidazole tablet/250mg (Indian)

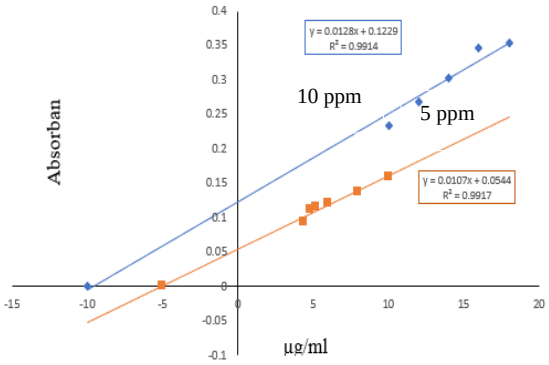


Fig.5 Standard addition method of Metronidazole intravenous injection500 mg/100ml (Iraq-Sulaymaniyah)

Table 11: results of standard addition method

Pharmaceutical dosage forms	µg/ml present	found	Recovery %	Error %
Metronidazole tablet/250mg (Indian)	5	4.692	93.858	-6,142
	10	10.027	100.27	+0.27
Metronidazole intravenous injection500 mg/100ml (Iraq-Sulaymaniyah)	5	5.084	101.682	+1.682
	10	9.602	96,015	-3.985

Table 12 :Determination of MZOL in human blood samples

Administration period /hour	Absorbance/ml of reduced samples*		Amount/found of extracted and diluted blood samples	Found µg/ ml	Average** (µg/ ml)	µg/25ml	Amount found (µg/3ml) blood***	Amount found (µg/ml) blood	RSD*%
4	2	0.071	0.4389(µg/2ml)	0.2194	0.24105	6.0262	100.035	33.345	±1.922
	4	0.17	1.0509(µg/4ml)	0.2627					
6	2	0.158	0.9767(µg/2ml)	0.4883	0.4770	11.925	197.955	65.985	±0.228
	4	0.308	1.9040(µg/4ml)	0.4760					
	6	0.453	2.8005(µg/6 ml)	0.4667					
12	2	0.096	0.5934(µg/2ml)	0.2967	0.2503	6.258	103.895	34.631	±0.442
	4	0.14	0.8654(µg/4ml)	0.2163					
	6	0.231	1.4280(µg/6ml)	0.238					
24	3	0.074	0.4574(µg/3ml)	0.1524	0.1302	3.255	54.046	18.015	±0.624
	6	0.127	0.7851(µg/6ml)	0.1308					

*Average of three replications, **A= 2.284x10^4 l. mol^{-1}.cm^{-1}x1cm x C mol. l^{-1}), ***Dilution factor:16.6

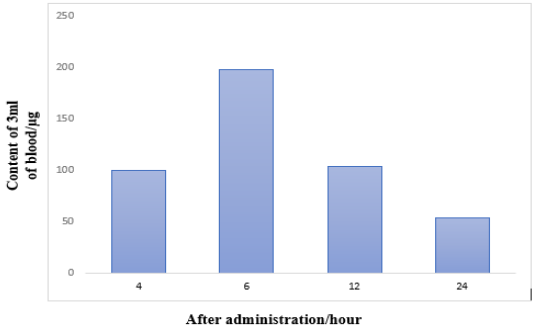


Fig.6 distribution of MZOL in blood after 4,6,12, and 24 hours after administration of Metronidazole 250mg tablet (Indian)

According to figure 6, MZOL is absorbed after oral administration, the drug exhibits maximum distribution in blood after 6 hours, the content in blood is reduced to its minimum value after 24 hours after administrated orally.

Comparison between the created method and literature methods for determination of MZOL

It is a smart, new, and unclassical way for determination of MZOL by diazotization of the reagent rather than the drug. Table 13 shows the higher sensitivity, and the wide applications range of the created method.

Table 13: Comparison between the created method and the update literatures methods for determination of MZOL

Parameter	Created method	Literature method [25]	Literature method [26]
• method	Diazotization of the reagent	Diazotization of MZOL	Oxidation-reduction
• Reagent	p-amino benzophenone	α-naphthol amine	Potassium permanganate
• Wavelength (nm)	431	510	610
• Beers law range (µg/ml)	2-24	2-12	4.28 – 59.91
• Limit of detection(µg/ml)	0.2407	0.1142	0.21
• Limit of quantification (µg/ml)	0.8024	0.3805	0.69
• Molar absorptivity ($l.mol^{-1}.cm^{-1}$)	$2.284x10^4$	$1.5x10^4$	$8.65x10^3$
• Sandell's sensitivity index ($\mu g\ cm^{-2}$)	0.0074	0.0114	0.019
• Application	Dosage forms Blood	Tablet	Dosage forms

4. Conclusion

This article offers a sensitive, precise method for determination of metronidazole in different dosage forms: tablet, injection, and suspension, at the same time it offers a precise method for estimation of metronidazole in blood after oral administration of metronidazole tablet at different periods of time which provide the ability of the follow up study of the drug in human blood with relative deviation not more than ±0.804 as an average.

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6. References

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