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Original Research Article

Development and Application of Kinetic Spectrophotometric Method for the Determination of Metronidazole

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Abstract

Purpose: *To develop an improved kinetic-spectrophotometric procedure for the determination of metronidazole (MNZ) in pharmaceutical formulations.*
Methods: *The method is based on oxidation reaction of MNZ by hydrogen peroxide in the presence of Fe(II) ions at pH 4.5 (acetate buffer). The reaction was monitored spectrophotometrically by measuring the rate of change of absorbance at 317nm.*
Results: *The optimum operating conditions for reagent concentrations and temperature were established. Linear calibration curve was obtained in the range of 85.77 – 513.45ng mL^{-1} with standard deviation from 1.77 to 4.55 %-. The optimized conditions yielded a theoretical detection limit of 15.20 ng mL^{-1} based on 3.3S_o criterion. Commonly used excipients and other additives such as talc, glucose, fructose, lactose, starch, magnesium stearate, microcrystalline cellulose and several ions were found not to have interference.*
Conclusion: *The developed method is sensitive, accurate and reproducible and could be used for routine analysis of metronidazole in pharmaceutical preparations*.

Keywords: Metronidazole, Kinetic spectrometry, Validation, Pharmaceutical preparation.

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INTRODUCTION

Metronidazole [2-(2-methyl-5-nitro- 1H- imidazol-1-yl) ethanol] is an amebicide, antiprotozoal and antibiotic effective against anaerobic bacteria and certain parasites.[1] It is the drug of choice for first episodes of mild-to-moderate *Clostridium difficile* infection [2]. Metronidazole exerts rapid bactericidal effects against anaerobic bacteria, with a killing rate proportional to the drug concentration. Concentration-dependent killing has also been observed with *Entamoeba histolytica* and *Trichomonas vaginalis*. Metronidazole kills *Bacteroides fragilis* and *Clostridium perfringens* more rapidly than clindamycin [3,4].

Review of literature for MNZ analysis revealed that several existing methods including different techniques such as HPLC with UV detection [5-7], GC-FID [8], HPLC PDA/MS [9] ,UPLC-MS [10] assay for its quantification in plasma and gastric juice fluids have been reported for assay of metronidazole.

There are UV-Vis spectrophotometric methods for the determination of metronidazole, but they

are not easy to perform, are not sufficiently sensitive, accurate and selective.

The aim of the present work, therefore, is to develop a simple, sensitive, specific, spectrophotometric method for the detection of MNZ in pharmaceutical tablet formulation, and that does not need sophisticated instruments or special skill, and requires less sample handling than methods currently described in the literature.

EXPERIMENTAL

Apparatus

Perkin-Elmer Lambda 15 UV/Vis spectrophotometer equipped with kinetic accessory provided with a temperature controlled cell.

Reagents

Stock solution ($1.0 \cdot 10^{-3}$ mol L^{-1}) of metronidazole was prepared in absolute ethanol from MNZ powder (certified purity: 99.92 %), kindly provided by Galenika, A.D., Belgrade, Serbia. The solution was stored at 4 ºC.
Analytical reagent grade chemicals and deionised water (MicroMed high purity water system, TKA Wasseraufbereitungssysteme GmbH) were used for the preparation of all solutions. Stock solution of Fe(II) ($1.0 \cdot 10^{-3}$ mol L^{-1}) was prepared by dissolving $FeSO_4 \cdot 7H_2O$ (Merck) in water. The pH of the reaction mixture was kept constant by adding acetate buffer prepared by standard procedure.

Hydrogen peroxide solution (0.1 mol L^{-1}) was prepared from the 34 % reagent.

All the glassware used were washed with aqueous HCl (1:1) and then thoroughly rinsed with running, distilled water, and then finally with deionised water.

General procedure

In order to obtain good mechanical and thermal stability, the instruments were run for 10 min prior to the first measurement. The reaction was carried out as follows. In the reaction mixture vessel with four compartments, the solution of MNZ was placed in one compartment, acetate buffer in the second, in the third, Fe(II), hydrogen peroxide and water (total volume: 10 mL) in the fourth compartment
.
The vessel was thermostated at 25.00 ±0.1°C and the reaction was initiated by vigorously shaking the reactants. The reaction solution was transferred to a cell, and the absorbance at 317 nm was measured spectrophotometrically every 30 s over a period of 5 - 6 min (after mixing) against the reagent blank prepared similarly. The rate of the reaction (*dc*/*dt*) at different concentrations of each of the reactants was obtained by measuring the slope of the linear part of the kinetic curves of the absorbance time plot. Based on Beer`s law, Eqs 1 and 2 were derived.

$A = \varepsilon \cdot l \cdot c$ ..... (1)

$tg\alpha = dA/dt = \varepsilon \cdot l \cdot dc/dt$ ..... (2)

where dc is the change in the concentration of indicator substances,
ε is molar absorption coefficient,
l - the thickness of the absorption layer and dA is the change in the absorbance of the indicator substance.

The calibration graph was constructed by plotting the slope of the linear part of the kinetic curve versus the concentration of MNZ [11].

Procedure for tablets

A total of twenty tablets of each of the different pharmaceutical formulations containing MNZ were weighed and finely powdered using a mortar and pestle. An accurately weighed portion of the resulting powder, equivalent to 10 mg of MNZ, was dissolved in 25 mL of ethanol. The mixture was centrifuged at 3500 rpm for 5 min, filtered through a 0.45 µm membrane filter (Millipore) directly into a 50 mL volumetric flask and made up to volume with ethanol to obtain a solution of theoretical MNZ concentration of 200 $ngmL^{-1}$. Aliquots of this solution were transferred into vessels spanning the concentration range listed in Table 4. In all cases, it was assumed that the actual content of the tablet corresponds to the labeled strength of the products.

Data were reported as mean ± standard deviation (SD) for five determinations. Statistical analysis was performed by Student t-test and F-test at 95 % confidence level, using a statistical package (Statistica 8.0, StatSoft, Inc, Tulsa, OK, USA) [11].

RESULTS

Kinetic studies

A differential variant of the tangent method [12] was used for the processing of the kinetic data because a linear correlation exists between the absorbance at 317 nm and time during the first 6 min after mixing. In order to determine the lowest possible determinable concentration of MNZ, the

conditions needed to be optimized. Therefore, the dependencies of the rates of the substrate reactions on the concentration of each of the reactants were determined.

Effect of variables

Keeping all other experimental parameters constant, the dependence of the reaction rate on pH in acetate buffer in the range of 3.0-5.0 was studied. The optimal difference between the rates of non-substrate and substrate reactions occurred at pH 4.5 and for further work, this pH value was selected because at higher pH values, the rate of both reactions significantly increased and thus, enlarged errors during measurement. For further work, a volume of 1.0 mL of acetate buffer pH 4.5 was selected as optimal.

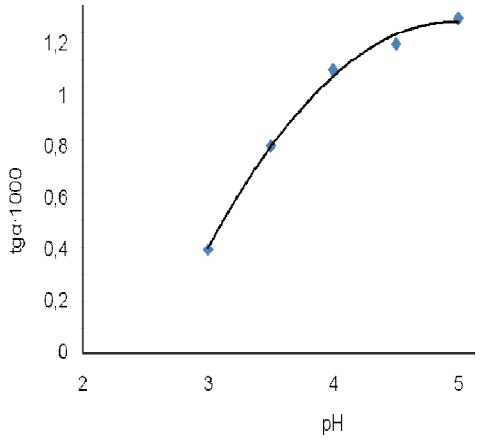

Figure 1: *Dependence of reaction rate on pH. Initial concentrations: C_{MNZ} = 2 x 10^{-7}mol L^{-1}, $C_{Fe(II)}$ = 1 x 10^{-6} mol L^{-1}, C_{H2O2} = 1.96 x 10^{-3} mol L^{-1}, t = 25 ± 0.1 °C, λ = 317 nm*

The correlation between the reaction rate and pH was not linear. Thus, the logarithms of tanα were calculated and the obtained values were plotted *vs* pH. From the obtained regression equations, the order of reaction was determined and was -0,3.

The dependence of reaction rates on the concentrations of Fe(II) were investigated over the range from 0.5 x 10^{-5} to 2.5 x 10^{-5} *mol* L^{-1}. For further work, a concentration of Fe(II) of 1 x 10^{-5} *mol* L^{-1} was selected as the optimal value, because at higher concentrations absorbance significantly increased (A > 1), which also increased the error of the spectrophotometric determination. The rate of substrate reaction was pseudo first order with respect to Fe (II) concentration.

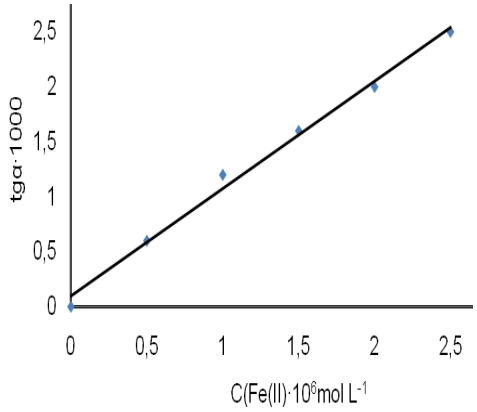

Figure 2: *Dependence of reaction rate on Fe(II) concentration. Initial concentrations: C_{MNZ} = 2·10^{-7} mol L^{-1}, C_{H2O2} = 1.96 x 10^{-3}mol L^{-1}, pH = 4.5 (acetate buffer), t = 25 ± 0.1 °C, λ = 317 nm,*

The dependence of reaction rate on concentration of H_2O_2 was investigated in the range 0.49 x 10^{-2} to 2.5 x 10^{-2} *mol* L^{-1}.

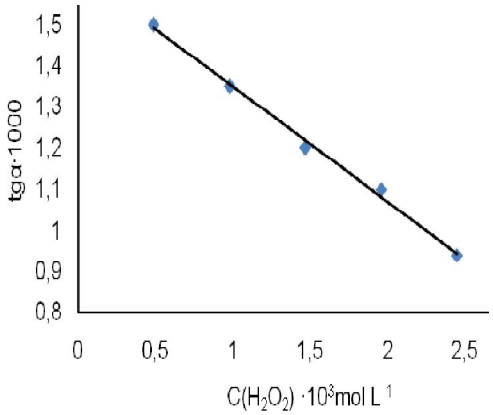

Figure 3: *Dependence of the reaction rate on H_2O_2 concentration. Initial concentrations: C_{MNZ} = 2 x 10^{-7} mol L^{-1}, pH = 4.5 (acetate buffer), $C_{Fe(II)}$ = 1 x 10^{-6} mol L^{-1}, t = 25 ± 0.1 °C, λ = 317 nm*

For further work, a concentration of H_2O_2 of 1.96 x 10^{-3} *mol* L^{-1}, was selected as a suitable value. The reaction rate at that concentration was optimal, and the kinetic curve was linear in the tested interval.

The rate of substrate reaction was (-1) order with respect to H_2O_2 concentrations in the investigated interval.

The optimal reaction conditions were:

pH = 4.5 (acetate buffer), $C_{Fe(II)}$ = 1 x 10^{-6} *mol* L^{-1}, C_{H2O2} = 1.96 x 10^{-3} *mol* L^{-1}, t = 25±0.1°C, *λ=317nm.*

MNZ concentrations were varied from 85.77 to 513.45 ng mL^{-1}, and a linear dependence was established between tg α and the concentration of MNZ . [12]

$$tg\alpha = 0.003\ C_{MNZ} - 0.017, \qquad R^2 = 0.998 \ldots\ldots\ldots (4)$$

where tgα is the slope of the linear part of the kinetic curve of the absorbance-time plot; C_{MNZ} is concentration of MNZ expressed as ng mL^{-1} and R is correlation coefficient. This equation was used for the determination of MNZ concentration in the interval mentioned.

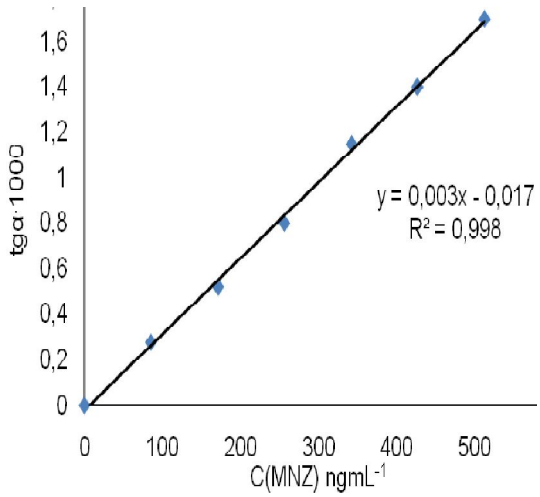

Figure 4: *Dependence of reaction rate on t MNZ concentration. Initial concentrations: pH 4.5 (acetatni pufer),* $C_{Fe(II)}$ *= 2 x 10^{-6} mol L^{-1},* C_{H2O2} *= 1,96 x 10^{-3} mol L^{-1}, t = 25 ± 0.1 °C, λ = 317 nm.*

The kinetic equation (Eq 3) for the reaction was deduced on the basis of the kinetics of the indicator reaction proposed.[13]

$$-\frac{dc}{dt} = k \cdot c_{H^+}^{-0,3} \cdot c_{Fe(II)}^{1} \cdot c_{H_2O_2}^{-1} \cdot c_{MNZ}^{1} \quad .(5)$$

where k is the constant proportional to the rate constant of the reaction.

Parameter value

The limit of detection (LOD) [14] was evaluated using Eq 6.

$$c_L = \frac{3.3 \cdot S_o}{m} \quad .....(6)$$

where S_0 is the residual standard deviation of the calibration line, and m is the slope of the calibration line (analytical sensitivity). LOD was found to be 15.20 ng mL^{-1}. The limit of quantification (LOQ) was evaluated using Eq 7.

$$c_Q = \frac{10 \cdot S_o}{m} \quad ..... (7)$$

It was found to be 45.6 ng mL^{-1}. The LOD value indicates that the method was sensitive. The precision and accuracy of the proposed method was studied by performing the experiment five times at three different concentration levels (low, medium and high) of MNZ. The results are shown in Table 1.

Table 1: Accuracy and precision of the proposed method

Taken (ng mL^{-1})	Found (ng mL^{-1})	N	SD%	RSD%	$\frac{\bar{x}-\mu}{\mu}\cdot 100\%$
513.45	506.5	5	1.45	1.77	4.16
171.54	168.83	5	2.21	3.22	1.58
85.77	82.21	5	0.84	4.55	1.35

Found- mean value; taken- true value, N-number of determinations; SD-. standard deviation, RSD- relative standard deviation, accuracy of method

$$\frac{\bar{x}-\mu}{\mu}\cdot 100\%$$

Interference studies

To assess the selectivity of the method, a systematic study of the possible interferences by species accompanying MNZ in pharmaceuticals was carried out. The criterion of interference was fixed at 5 % variation of the average slope change measured (n = 5) for the established level of MNZ. The tolerance limits (expressed as w/w ratio) for the species studied on the determination of 171.54 ng mL^{-1} of MNZ are given in Table 2.

Table 2: Tolerance ratio for foreign species in the determination of MNZ(171.54ng mL^{-1}) under optimal conditions.

Tolerance level C_x/C_{MNZ}	Foreign species
100	Na^+, K^+, Ni^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, NO_3^-, CH_3COO^-, CO_3^{2-}, Cl^-
100	talc,glucose, fructose, lactose, starch, magnesium stearate, microcrystalline cellulose
10	Se^{4+}, Cd^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, Br^-, SO_4^{2-},
1interfere	ascorbic acid, citric acid, doxicyclin, streptomycin,

Commonly used excipients and other additives such as talc, glucose, fructose, lactose, starch, magnesium stearate, microcrystalline cellulose and several ions were found to have no interference. More severe interference was observed for ascorbic acid, citric acid and other antibiotics in the ratio 1:1.

Applicability of the proposed method

In order to test the analytical validity of the proposed method, it was applied to the

Table 3: Determination of MNZ in commercial tablet formulation by the proposed method (kinetic) and standard potentiometric) methods

Pharma-ceutical prep.	Found MNZ kinetic meth. (ng mL^{-1})	RSD (%)	Recovery (%)	Found MNZ Standard meth (ng mL^{-1})	RSD (%)	Recovery (%)	F-test*	t-test
Flagil	198.63±2.30	1.18	98.20	198.80±2.06	1.03	97.89	3.26	1.83
Orvagil	197.25±3.12	1.56	97.76	198.71±2.26	1.13	98.35	3.59	1.73
MNZ Normon	197.83±2.86	1.43	98.54	198.65±2.48	1.24	99.20	4.02	2.06

*Data are based on the mean of five determinations; *theoretical F-value (v1 = 4, v2 = 4) and t-value (v = 8) at 95 % confidence level are 6.39 and 2.306, respectively*

Table 4: Comparison of proposed method with existing spectrophotometric methods

Method	Reagents	Λ_{max}	Restriction of method	Ref
1	3-Methyl benzothiazolin-2-one hydrazone (MBTH)	500	MBTH is costly reagent	[16]
2	N-1-naphthyl-ethylenediamine dihydrochloride (NEDA)	520	Involves the addition step of diazotization	[16]
3	*p*-Dimethylaminocinnamaldehyde	510	Condenzation reaction is time consuming	[17]
4	β-Naphthol	480	Three step procces:reduction, diazotization, coupling	[18]
5	Metal and $K_2Cr_2O_7$	502	Involves reduction by Zn-HCl at pH 2,9. Color formation and stability depend of pH value	[19]
6	NN-dimethyl-*p*-phenylenediamine and chloramine-T	540	Involves reduction by Zn-HCl at pH 7. Color formation and stability depend of pH value	[20]
7	Bromocresol green	654	Involves extraction with $CHCl_3$, at pH 9,5	[21]
8	Bromocresol green purple	618	Involves extraction with $CHCl_3$, at pH 10	[21]
9	8-Quinolinol	500	Reagent is costly and not easily available.	[22]
10	*p*-Benzoquinone	526	Two step proces,reagens is cheep and easily available	[23]
11.	***This work***	317	Fast, one step proces, high sensitivity, UV-detection	

determination of MNZ in pharmaceutical formulations.

The results of the proposed method were statistically compared with those of the standard potentiometric method [15] using a point hypothesis test.
Statistical analysis of the results (Tables 3.) showed that calculated F- and t-values at 95 % confidence levels were less than the theoretical ones, confirming no significant differences between the performance of the proposed and the standard potentiometric method. Therefore, the proposed method could be used for the determination of MNZ in pharmaceutical preparations.

DISSCUSION

The method is based on the reaction of MNZ by hydrogen peroxide in acetate buffer (pH 4.5), in the presence of Fe(II), which acted as a catalyst. The reaction was monitored spectrophotometrically by measuring the absorbance of formed product at 317 nm.
Under optimal conditions (pH 4.5 (acetate buffer), $C_{Fe(II)}$ = 1 x 10^{-6} *mol* L^{-1} , C_{H2O2} = $1.96 \cdot 10^{-3}$ *mol* L^{-1}, t = 25 ± 0.1 °C, λ = *317 nm),* the method showed statisfactory standard deviation and relative standard deviation (from 1.77 to 4.55 %), respectively. Least-squares regression analysis used to evaluate the concentration range data indicates linearity over the interval studied (85.77 ng mL^{-1} to 513.45 ng mL^{-1}). The correlation coefficient obtained for this MNZ concentration range was 0.998. The LOD

value of 15.20 ng mL^{-1} indicates that the method is sensitive. Commonly used excipients and many investigated ions were found to have no interference.

The results of the proposed method were statistically compared with those of the standard potentiometric method method using a point hypothesis test. Statistical analysis of the results showed that calculated F- and t-values at 95 % confidence levels were less than the theoretical ones, confirming no significant differences between the performance of the proposed and the standard method. Therefore, the proposed method could be used for the determination of metronidazole in pharmaceutical preparations. A comparison of the present method with the existing spectrophotometric methods is given in Table 4. This demonstrates the advantages of the proposed method.

CONCLUSION

The proposed kinetic-spectrophotometric method for the determination of MNZ in pharmaceutical samples reported in this work is simple, fast, inexpensive, and thus appropriate for routine quality control analysis of the active drug in the laboratories of hospitals, pharmaceutical industries and research institutions. It should also be suitable for developing countries. The validation of the method shows that the results obtained are in good agreement with the potentiometric method.

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