Spectrophotometric Method for the Determination of Metronidazole in Pharmaceutical products based on the Formation of Azo dye

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ABSTRACT

Two simple, rapid, sensitive and accurate spectrophotometric methods have been developed for the estimation of metronidazole. The method depends on the reduction of Metronidazole molecule with zinc powder and hydrochloric acid followed by diazotization and coupling with 2-hydroxybenzaldehyde to give yellow colored in alkaline medium with an absorption maximum at 457 nm. Under optimized experimental conditions Beer's law was obeyed in the concentration ranges 3.0 to 15.0 μg/ml and 25.0 to 37.0 μg/ml. Also, by using p-aminoacetophenone as a coupling agent to form orange colored with an absorption maximum at 490 nm. Beer's law was obeyed in the concentration ranges 3.0 to 40.0 μg/ml. The Sandell's sensitivity, the molar absorptivity, correlation coefficient, detection limit and the regression equation were calculated. Both techniques were applied successfully to a wide variety of pharmaceutical preparations.

1. INTRODUCTION

Metronidazole is commonly known by the trade name Flagyl (O'halloran et al., 2010). It is chemically named 2-(2-methyl-5-nitro-1-H-imidazole-1-yl)-ethanol (Klimowicz et al., 2002). With chemical formula (C₆H₉N₃O₃), molecular mass 171.12 g/mol] (Dharuman, 2007), and the chemical structure is shown in (Fig. 1) (Klimowicz et al., 2002). It is white or pale yellow crystalline powder (Tyagy, 2008). With a slight odor and a bitter slightly saline taste (Al-sabea, 2008). Slightly soluble in organic solvents unstable at room temperature.
turns dark on exposure to light. It is stable in air, but is light-sensitive (Tyagy, 2008). It was discovered in the 1950s. In the 1980s, it becomes fully established that it has powerful antibacterial activities against the anaerobic bacteria (Chaudhuri, 2009). Several methods have been reported for the determination of metronidazole in pharmaceutical products which includes Spectrophotometry (Youssef et al., 2015) & (Masood et al., 2016), HPLC (Pai and Pusalkar, 2014) & (Budiarti et al., 2015), RP-HPLC (Rajendar et al., 2015) & (Kumar et al., 2015), gas chromatography flame ionization detector (GC-FID) (Ashour and Kattan, 2010). flame atomic absorption spectrophotometry (FAAS) (Abdul-ghani et al., 2012). Most of the spectrophotometric methods reported suffer from the disadvantage, like narrow range of determination, requires heating or extraction, long time for the reaction to complete, use of non-aqueous systems and stability of the colored product formed, etc. The purpose of our present investigation is to develop and validate a new simple, selective accurate and rapid spectrophotometric assay for the determination of Metronidazole in pharmaceutical formulations.

![Chemical structure of metronidazole](image)

Figure (1): Chemical structure of metronidazole.

2. MATERIALS AND METHODS

Apparatus

Metronidazole spectra and measurement were recorded using an Agilent Technologies Cary Series UV-Vis double beam Spectrophotometer product No. (G9821A), Serial No. (MY13140002). A 1-cm quartz cell was used in the present work. To obtain pH readings throughout the experimentation, a microprocessor pH meter (HANNA HI 211) was used.

Reagents and samples

All chemicals used in the study were of analytical grade; Methanol and distilled water were used as solvent for preparation of solutions throughout the experiments.

Reagents

2-Hydroxybenzaldehyde (2-HBA) (1.0%) was prepared by diluting (1.0ml) of concentrated 2-hydroxybenzaldehyde (purity % =99.9) to 100ml methanol.

p-Aminoacetophenone (PAAP) (1.0%) was prepared by dissolving 1.0g of p-aminoacetophenone and completed to 100ml with methanol.

Standard stock Solution

Metronidazole (MN) stock solution (1000 μg/ml) the metronidazole solution (5.844×10⁻³ M) was prepared by dissolving 0.10 g of MN and diluted to 100 ml with methanol.

Reducing of metronidazole (RMN) freshly reduced metronidazole (RMN) was prepared by adding 0.1gm of zinc dust and 3.0ml of concentrated hydrochloric acid to 10ml of stock solution of MN, then diluted to 70ml and refluxed up to 75°C for 15 minutes with stirring, after cooling, the solution was filtered and completed to the volume in a 100 ml volumetric flask with distilled water to obtain the standard solution with concentration of 100 ppm (Alsamarrai, 2011).

Sample preparations

Seven different brands of pharmaceutical products were used (contains 500,250, 200 mg MN) metronidazole were weighed and ground into a fine powder then mixed thoroughly. An accurately weighed amounts of
the powder mixture that contain 0.1 g MN (0.0345 g powder brand A, 0.0374 g powder brand B, 0.0384 g brand C, 0.0352 g brand D, 0.0427 g brand E, 0.0473 g brand F, 0.0345 g brand G) were mixed with 40 ml of methanol and stirred for 10 min to increase solubility. The insoluble mass was filtered off on a Whatman No.41 filter-paper, washed with methanol and the filtrate plus washings was diluted to 100 ml with methanol in a volumetric flask (Shah et al., 2005).

**Preliminary test for determination of λ max of RMN-2-HBA**

10.0 ml of 0.1N of hydrochloric acid solution and 0.5 ml of 1.0% of sodium nitrite solution were mixed well then 3.0 ml from (100 μg/ml) of RMN solution at room temperature was added to the mixture in 10 ml volumetric flask. The solution was shaked for 1 min, 1.0 ml of 0.1M sodium hydroxide solution and 1.0 ml of 1.0% 2-hydroxybenzaldehyde solution were added and mixed well. The azo dye solution was then scanned in the range (400-600nm) from the spectrophotometer; the wavelength of maximum absorption selected for determination of RMN-2-HBA was 455 nm. (Fig. 2) shown the maximum absorption spectra of RMN-2-HBA and the blank have low absorbance value at this wavelength.

**Recommended procedures for determination of metronidazole using 2-HBA reagent**

To a series of 10 ml volumetric flasks, 0.1 ml of 0.1 N hydrochloric acid solutions with 2.5 ml of 1.0% sodium nitrite solution were added and mixed well. Then aliquot of RMN solution containing (3.0-15.0 μg/ml) or (25.0-37.0 μg/ml) at room temperature was added to the mixture. The solution was shaked for 2 min and 1.0 ml of 0.1M sodium hydroxide solution and 1.0 ml of 1.0% 2-HBA solution were added, mixed and then diluted to the mark with distilled water. The absorbance was measured directly at 457 nm against reagent blank prepared in similar conditions without RMN.

**Preliminary test for determination of λ max of RMN-PAAP**

A 37.0 μg/ml from working solution of RMN (100 μg/ml) was mixed well to 1.0 ml of 0.1N hydrochloric acid solution 1.5 ml of 1.0% sodium nitrite solution, in ice bath in 10 ml volumetric flask. The solution was shaked for 2 min. 1.0 ml of 1.0% of PAAP and 1.0 ml of 0.1 M sodium hydroxide solution were added then mixed well in the ice bath for 5 min. The solution diluted to the mark with distilled water and the azo dye solution was then scanned in the range (300-550 nm) from the spectrophotometer; the wavelength of maximum absorption selected for determination of RMN-PAAP was 488 nm. (Fig. 3) shown the maximum absorption spectra of RMN-PAAP and the blank have low absorbance value at this wavelength.

![Figure (2): Absorption spectra of (a) azo dye RMN-2-HBA against blank, (b) blank against distilled water.](image-url)
Figure (3): Absorbance spectra: (a) azo dye RMN-PAAP against blank solution (b) the blank solution against distilled water (c) PAAP reagent against methanol.

Recommended procedures for determination of metronidazole using PAAP reagent

To a series of 10 ml volumetric flasks, 0.5 ml of 0.1N Hydrochloric acid solution with 1.0 ml of 1.0% of sodium nitrite solution were added and mixed well. Then an aliquots of RMN solution containing (3.0-40μg /ml), at <5 °C in ice bath was added to the mixture. The solution was shaked for 2 min and 1.0 ml of 0.1 M sodium hydroxide solution and 0.5 ml of 1.0% of reagent (PAAP) were added to produced azo dye compound and the final solution mixed well in ice bath for 5 min and then diluted to the mark with distilled water. The absorbance was measured at 490 nm against reagent blank prepared in similar conditions without RMN.

3. Results and Discussion

Optimization of experimental parameters

All chemical conditions that participate in the reaction have been studied to obtain maximum sensitivity for both methods; therefore, different variable conditions were investigated to obtain optimum conditions. A univariate method was used for this purpose.

Effect of acid types

Different types of acids (0.1N) have been tested for maximum absorption achievement. The hydrochloric acid solution gave best results in contrast with other acids as shown in (Table 1).

Table 1: Effect of different of acids

| Acid types   | Absorbance     |
|--------------|----------------|
|              | First method   | Second method |
| HCl          | 0.5687         | 0.5193        |
| H₂SO₄        | 0.4922         | 0.2530        |
| CH₃COOH      | 0.0132         | 0.1740        |
| HNO₃         | 0.0446         | 0.3347        |

The effect of the different volumes of hydrochloric acid solutions (0.1N) was examined in both methods. The absorbance increased by increasing volume of the acid, after which there is an apparent decreasing in absorbance in higher volumes because the excess of acid converts the diazonium ion to diazonium salts (Ar-NH₃⁺Cl⁻) (Morrison and Boyd, 2002). The results show in (Table 2).

Table 2: Effect of the volume of hydrochloric acid.

| Volume of 0.1N hydrochloric acid solution(ml) | Absorbance     |
|---------------------------------------------|----------------|
|                                            | First method   | Second method |
| 0.05                                        | 0.5587         | 0.1032        |
| 0.1                                         | 0.5697         | 0.2031        |
| 0.3                                         | 0.5423         | 0.2474        |
| 0.5                                         | 0.5120         | 0.5371        |
| 1.0                                         | 0.4887         | 0.5193        |
| 1.5                                         | 0.4599         | 0.4534        |

Effect of 1.0% sodium nitrite

Sodium nitrite solutions with different volumes have been used to study the effect on
the formation of azo dye. And it was used in the subsequent experiments to converted NH₂ group to a diazonium (Morrison and Boyd, 2002). The results of both methods show in (Table 3).

Table 3: Effect of sodium nitrite solution.

| Volume of 1.0% sodium nitrite solution | Absorbance First method | Absorbance Second method |
|---------------------------------------|-------------------------|--------------------------|
| 0.3                                   | 0.0425                  | 0.0412                   |
| 0.5                                   | 0.5698                  | 0.3453                   |
| 1.0                                   | 0.5788                  | 0.5532                   |
| 1.5                                   | 0.5789                  | 0.5424                   |
| 2.0                                   | 0.5865                  | 0.4674                   |
| 2.5                                   | 0.5912                  | 0.4411                   |
| 3.0                                   | 0.4985                  | 0.3618                   |
| 3.5                                   | 0.4460                  | 0.3054                   |

Effect of time on the formation of diazonium ion

The colored azo dye reached its maximum intensity when the coupling reagent was added to the diazonium ion solution after standing for 2 minutes.

Effect of the amount of 2-HBA

The effect of different volumes of 1.0% 2-HBA solution was examined on the maximum formation of the colored azo dye (Table 4) shows that 1.0 ml of the solution is enough to obtain the maximum absorption, and it was used in the subsequent experiments.

Table 4: Effect of the amount of 2-HBA solution.

| Volume of 1.0% of 2-HBA solution | Absorbance First method |
|----------------------------------|-------------------------|
| 0.1                              | 0.1114                  |
| 0.3                              | 0.2901                  |
| 0.5                              | 0.5155                  |
| 1.0                              | 0.6452                  |
| 1.5                              | 0.5406                  |
| 2.0                              | 0.4981                  |
| 2.5                              | 0.4712                  |

Effect of the amount of PAAP

The influence of the different volumes of 1.0% PAAP on the maximum color development of the azo dye was studied. The results show in the (Table 5) that 0.5 ml of the solution is enough to obtain the maximum absorbance value, and it was used in the subsequent experiments.

Table 5: Effect of volume PAAP solution

| Volume of 1.0% of PAAP solution | Absorbance Second method |
|---------------------------------|--------------------------|
| 0.1                             | 0.1237                   |
| 0.3                             | 0.2802                   |
| 0.5                             | 0.5823                   |
| 1.0                             | 0.5532                   |
| 1.5                             | 0.4939                   |
| 2.0                             | 0.4420                   |

Effect of the base types

For the both methods the effect of different types of bases (sodium hydroxide, potassium hydroxide and sodium carbonate) on coupling reaction of diazonium ion with the reagents was investigated. At 1.0 ml of 0.1M of sodium hydroxide solution more suitable and gave maximum absorption among other bases.

Effect of the temperature
It is well-known that diazonium ion stability is affected by temperature (Bruice, 2004). The diazotization reaction was carried out in ice bath and at room temperature. In the first method there was no different in the measurements of the absorbance of the azo dye, with or without using ice, so, subsequent experiments were studied at room temperature. But in the second method the reaction gave maximum absorption in ice bath.

4. Validity of the analysis method

Calibration curve

Under optimum conditions in the first method, two calibration curves were constructed. First when a straight line was obtained as shown in (Fig. 4), the calibration curve was obeyed Beer’s law in the concentration range of 3.0-15.0 μg/ml with detection limit 0.5 μg/ml. The second one when a straight line was obtained as shown in (Fig. 5), the calibration showed linearity in the concentration range 25.0-37.0 μg/ml. The optical and statistical characteristics of the method are shown in (Table 6). From the results of both calibrations. But in the second method under optimum conditions the calibration curve was obtained as shown in (Fig. 6). The calibration showed good linearity in the concentration range (3.0-40) μg/ml of RMN, and (Table 7) shows optical characteristics of the method.

Table 6: The statistical data of the calibration curves obtained using spectrophotometric determination of RMN-2HBA.

| Parameter                     | First calibration | Second calibration |
|-------------------------------|-------------------|--------------------|
| λ max (nm)                    | 457               | 457                |
| Colour                        | Yellow            | Yellow             |
| Beer’s law (μg/ml)            | 3.0-15.0          | 25.0-37.0          |
| Detection limit (μg/ml)       | 0.5               | ……….             |
| coefficient of determination,R²| 0.9941            | 0.9993             |
| Molar absorptivity (L/mol.cm) | 3.097×10³         | 3.405×10³          |

Figure 4: First calibration curve of the determination of RMN -2HBA.

Figure 5: Second calibration curve of the determination of RMN-2-HBA.

Figure 6: Calibration curves for determination of RMN using PAAP.
Table 7: The statistical data of the calibration curve obtained using spectrophotometric determination of RMN-PAAP.

| Parameter                        | Characteristic |
|----------------------------------|----------------|
| λ max (nm)                       | 490            |
| Colour                           | Orange         |
| Beer’s law (μg/ml)               | 3.0 – 40.0     |
| Detection limit (μg/ml)          | 0.5            |
| Coefficient of determination, R² | 0.9996         |
| Molar absorptivity (L/mol.cm)    | 2.6352×10³     |
| Sandell index(μg/cm²)            | 0.05           |

Accuracy and precision

The precision and accuracy of the determination of reduced metronidazole were studied depending upon the values of the relative standard deviation percentage (RSD%), and relative error percentage (E_rel %) for five replicates, respectively.

Study of interferences

Synthetic solutions were prepared in order to study the effect of the interferences of various compounds. Various concentrations of different compounds were added individually to the solutions containing 32.0 μg/ml of reduced metronidazole (RMN). Then the recommended procedure was applied. That causes errors of ≤ ±5.0%.

Application of the method

The recommended spectrophotometric methods when using 2-HBA and PAAP reagents, was successfully applied for the determination of MN in various pharmaceutical products. The data were compared with those obtained by standard method (HPLC) from Awamedica Company for drugs in Erbil-Iraqi Kurdistan region which depend on British pharmacopeia.

5. Conclusions

In the present work spectrophotometric method have been used for determination of metronidazole using new reagents depending upon diazotization coupling reaction. Simple, new and sensitive spectrophotometric methods for determination of metronidazole in pharmaceutical products have been proposed based on the azo coupling reactions, by using two new reagents (2-hydroxybenzaldehyde) and (p-aminoacetophenone). The main feature of the present methods is simplicity, low cost. The procedures for metronidazole analysis in pharmaceutical products are free from tedious steps like extraction, complex sample treatment and more heating. (Table 8) shows a comparison of metronidazole determination with other methods.
Table 8: Comparison of the proposed method for spectrophotometric determination of metronidazole with other methods.

| Reagent               | λ_{max} (nm) | Linearity (μg/ml) | D.L (μg/ml) | Molar absorptivity (Lmol^{-1}cm^{-1}) | Ref.                                         |
|-----------------------|--------------|-------------------|-------------|---------------------------------------|---------------------------------------------|
| Chloranilic acid      | 520          | 5-40              | 1.88        | 2.74 \times 10^3                      | (Adegoke et al., 2010)                      |
| Diloxanide furoate    | 314          | 4-24              | ……          | 3.24 \times 10^3                      | (El-Ghobashy and Abo-Talib, 2010 )         |
| Furazolidone          | 320.2        | 5-30              | ……          | 0.0575                                | (Chemate et al., 2012)                      |
| Ciprofloxacin         | 295          | 2.0-10.0          | ……          | 4.3 \times 10^3                       | (Patel and Prajapati, 2012 )               |
| Diiodohydroxyquinoline| 510          | 2.0-10            | ……          | 9.9 \times 10^3                       | (Ali et al., 2013 )                        |
| 2-hydroxybenzaldehyde| 457          | 3.0 - 15.0        | 0.5         | 3.0971 \times 10^3                    | Proposed work                              |
| P aminoacetophenone   | 490          | 3.0 – 40.0        | 0.5         | 2.6352 \times 10^3                    |                                             |

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REFERENCES

AL-SABEA, N. (2008). "Assay of Metronidazole from Different Manufacturing Sources in Iraqi Markets " AJPS, 5(1): pp. 17-18.

ASHOUR, S. & KATTAN, N. (2010). "Simultaneous determination of miconazole nitrate and metronidazole in different pharmaceutical dosage forms by gas chromatography and flame ionization detector (GC-FID)." Int J Biomed Sci, 6(1): pp. 13-18.

ABDUL-GHANI, A. J., JASIM, H. H. & AL-KADUMI, A. S. H. (2012). Molecular and atomic spectrophotometry and high performance liquid chromatographic determination of of metronidazole in dosage forms via complex formation with Au(III) and Hg(II) ions in Solutions. Journal of Chemical and Pharmaceutical Research, 4(7): pp. 3749-3758.

ALSAMARRAI, K. F. (2011). "Spectrophotometric Determination of Metronidazole via Diazotization Reaction with p-Hydroxy Benzaldehyde as a Coupling Reagent." Kerbala Journal of Pharmaceutical Sciences, 2: pp. 48-58.

ADEGOKE, O. A., UMHOA, O. E. & SOYINKAB, J. O. (2010). "Spectrophotometric determination of
metronidazole and tinidazole via charge transfer complexation using chloranilic acid.” Journal of the Iranian Chemical Society, 7(2): pp. 359-370.

ALI, N. W., GAMAL, M., ABDELKAWY, M. (2013) Spectrophotometric determination of Diodohydroxyquinoline in presence of Metronidazole in pharmaceutical formulation. International Journal of Analytical and Bioanalytical Chemistry, 3(2): pp. 52-58.

BUDIARTI, A., GANDJARB, I. & ROHMAN, A. (2015). "Liquid Chromatography With UV Detection For Simultaneous Determination Of Ciprofloxacin And Metronidazole." Jurnal Teknologi, 72(1): pp. 45-47.

BRUCE, P. Y. (2004). Organic chemistry. 5th ed., Prentice Hall India, pp. 684-688.

CHAUDHURI, S. K. (2009). Quintessence of Medical Pharmacology, New Central Book Agency: p. 291.

CHEMATE, S. Z., DONGARE, U. S., JADHAV, S. A. & JADHAV, M. B. (2012). "Validated spectrophotometric methods for simultaneous estimation of metronidazole and furazolidone in pure and in tablet dosage form.” Int. Res. J. Pharm, 3: pp. 461-464.

DHARUMAN, J. (2007). Pharmaceutical organic chemistry. India, Virender Kumarary: p. 35.

EL-GHOBASHY, M. R. & ABO-TALIB, N. F. (2010). "Spectrophotometric methods for the simultaneous determination of binary mixture of metronidazole and diloxanide furoate without prior separation." Journal of Advanced Research, 1(4): pp. 323-329.

KLIMOWICZ, A., BIELECKA-GRZELA, S. & TOMASZEWSKA, U. (2002). "A simple and rapid liquid chromatographic method for the determination of metronidazole and its hydroxymetabolite in plasma and cutaneous microdialysates." Acta poloniae pharmaceutica, 59(5): P. 327.

KUMAR, S. Y., SIVAPRSAD, P. & KUMAR, A. A. (2015). "RP-HPLC method development and validation for simultaneous quantitative estimation of metronidazole and nalidixic acid in tablets.” International Journal of Pharmacy and Pharmaceutical Sciences, 7(2): pp. 367-371.

MASOOD, Z., ANSARI, M. T., ADNAN, S., SAEED, M. S., FAROOQ, M. & AHMAD, M. (2016). "Development and application of spectrophotometric method for quantitative determination of Metronidazole in pure and tablet formulations." Pakistan Journal of Pharmaceutical Research, 2(1): pp. 28-32.

MORRISON, R. & BOYD, R. N. (2002). Organic Chemistry. 6th ed. India: Prentice- Hall: p. 774.

O'HALLORAN, E., HOGAN, A. & MEALY, K. (2010). "Metronidazole-induced pancreatitis." HPB surgery: pp. 1,4.

PAI, N. R. & PUSALKAR, D. A. (2014). Development and validation of liquid chromatographic method for metronidazole. Pelagia Research Library, 5(1): pp.23-28.

PATEL, N. V. & PRAJAPATI, A. M. (2012). "Q-Absorbance Ratio Spectrophotometric Method for the Simultaneous Estimation of Ciprofloxacin and Metronidazole in their Combined Dosage Form.” JPSBR, 2(3): pp. 118-122.

RAJENDAR, L., NAGA, R. P. & NARSIMHA, R. (2015). A stability indicating RP-HPLC method for the simultaneous estimation of metronidazole, clindamycin and clotrimazole in bulk and their combined dosage form. World J Pharm Sci, 3(1): pp. 93-103.

SHAH, J., JAN, M. R. & KHAN, M. A. (2005). Determination of furosemide by simple diazotization method in pharmaceutical preparations. Journal of the Chinese Chemical Society, 52: pp.347-352.

TYAGY, A. (2008). Encyclopedic dictionary of pharmacy. India, Virender Kumarary: p. 333.

YOUSSEF, A. K., SALEH, M. M. S., ABDEL-KADER, D. A. & HASHEM, E. Y. (2015). "facile spectrophotometric determination of metronidazole and secnidazole in pharmaceutical preparations based on the formation of dyes." International Journal of Pharmaceutical Sciences and Research, 6(1): pp. 103-110.