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Spectrophotometric Methods for Determination of Metronidazole in Pharmaceutical Formulations

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A R T I C L E I N F O

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A B S T R A C T

Two accurate and reproducible spectrophotometric methods were developed for determination of metronidazole (MET). The methods are based on the reduction of metronidazole with iron metal and hydrochloric acid followed by coupling with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reagent to form a purple colored chromogen which was easily measured spectrophotometrically at 546 nm (method A).The proposed method B involves the reduction of metronidazole in the presence of tungstate and/or molybdate in an alkaline medium and then coupling of the reaction product with FCR to form a blue colored chromogen which was measured spectrophotometrically at 725nm.The optimized experimental condition were obeyed the beer's law with a good linearity over the concentration ranges 0.3-30 and 0.5 - 40µg/ml of metronidazole with both NBD-Cl and FCR respectively.The limit of detection (LOD) and limit of quantitation (LOQ) were 0.16 and 0.56 µg/ml for method A; while for method B were 0.05 and 0.18 µg/ml, respectively. The proposed methods were applied successfully for determination of MET in pharmaceutical formulations

1. INTRODUCTION

(MET) is a 5-nitroimidazole derivative that used as antiprotozoal, antiamebic and antibacterial drugs (Wade et al., 1977). Metronidazole, merchandised under the brand name Flagyl among others, is an antibiotic and antiprotozoal medication. It is utilized either alone or with other antibiotic to treat pelvic fiery malady, endocarditis, and bacterial vaginitis (The American Society of Health-System Pharmacists, 2015). Chemically, metronidazole is known as 2-methyl-5-nitroimidazole-1-ethanol and it belongs to nitroimidazole group (Remington and Gennaro, 1990). MET ($C_6H_9N_3O_3$, M.Wt= 171.156g/mole) is slightly soluble in water but soluble in alcohols and acetone and has a

chemical structure as shown in Fig 1.(British Pharmacopeia, 2013).

MET has been determined by potentiometric titration using 0.1M perchloric acid as a titrant(British Pharmacopeia on CD-ROM., 2013). Other methods have also been reported for the determination of MET in pharmaceutical formulation such as electrochemical methods (Kattab et al., 2011; Liu et al., 2013; Yilmaz et al., 2013; Sarr et al., 2016) , chromatography (Ouyang, 2010; Ashour and Kattan , 2010 ;Al-Halabi et al., 2012) , and flow injection analysis (Simoes et al., 2006).

Visible spectrophotometry remains competitive comparing with other methods for pharmaceutical analysis due to its simplicity, cost effectiveness, sensitivity, accuracy and precision. Initial reduction of the nitro group has been used as a basis for determination of metronidazole in the visible region followed by either diazotization and coupling of the resulting amine with different coupling agents (Ibrahim and Bashir, 2012; Youssef et al., 2015) or resulting amine undergoes a condensation reaction with aromatic aldehyde to form Schiff's bases (Siddappa et al., 2008). Other visible spectrophotometric methods based on different reaction schemes have been reported in literature for the determination of MET in pharmaceutical formulations (Thulasama and Venkateswarlu 2009 ; Miljkovic et al., 2014) . However, none of these methods are satisfactory, Some of these methods have narrow linear range (Saffaj et al., M., 2004), time consuming steps like heating and extraction (Lopez et al., 1997; Bhatkar and Chodankar, 1980), used costly reagents and include an additional diazotization step (Adegoke and Umoh, 2009), critical dependence on pH (Alsamarrai, 2011). Hence, there is a need to develop another method in order to minimize such disadvantages.

The developed spectrophotometric methods are based on the reduction of MET with powdered iron and HCl, followed by coupling with NBD-Cl or FCR. The experimental conditions were optimized and then Beer's law limits, Sandell's sensitivity and molar absorptivity were calculated for the proposed methods. The accuracy and precision of the developed methods were evaluated and the effect of some common excipients was studied. The standard potentiometric titration method (British Pharmacopeia on CD-ROM., 2013) was accomplished for comparing the present methods statistically, and it was found that the results of both methods were not significantly different.

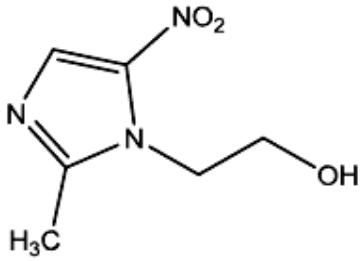


Fig.1. Chemical structure of metronidazole (MET)..

2.EXPERIMENTAL AND METHODS

2.1. Experimental

2.1.1. Apparatus

UV-VIS spectrophotometer (Cecil CE3021-England) was used for all spectral and absorbance measurements using 1.0 cm quartz cells. Thermostatically controlled water bath (Lab. companion shaking BS-11, Korea) was used for temperature control.

2.1.2. Reagents

All chemicals used in this study were of analytical grade. A pure metronidazole (MET) was obtained as gifts from (Awamedica Company for Drug Industries and Medical Applications Awa, Erbil). Folin-Ciocaleteu reagent (FCR) and 4-chloro-7-nitrobenzo-2-

oxa-1, 3-diazole (NBD-Cl) were purchased from Sigma Chemicals Co., USA. Sodium hydroxide and sodium bicarbonate were obtained from Fluka.

2.1.3. Solutions

- Reduced metronidazole solution (100 µg /ml) was prepared by dissolving 0.01g of MET in 10 ml deionized water followed by addition of 0.4 g of powdered iron and 5 ml of concentrated HCl.The solution was stirred for 20 min at room temperature and then filtered. The filtrate was diluted with deionized water to 100 ml in a volumetric flask and then kept in an amber-glass volumetric flask, where it was stable at least for 2 days (Al-Temur, 2015).

-Serial dilutions of reduced MET solution (100 µg /ml) with deionized water were made to cover the working range of the calibration graph.

- Folin-Ciocaleteu reagent (FCR)(1:3, v/v) was prepared by mixing accurately measured 25 ml of commercially available FCR in 75 ml of deionized water.

- Solution of 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) (0.3%) was freshly prepared by dissolving 0.3 g of NBD-Cl in 100 ml of acetone.

- Sodium hydroxide solution (2 M) was prepared by dissolving 8 g of sodium hydroxide in deionized water and then diluted to the mark with deionized water in 100 ml volumetric flask.

- Sodium bicarbonate solution (2 M) was prepared by dissolving 16.8 g of the pure sodium carbonate in deionized water and then filtered .The filtrate was diluted to the mark with deionized water in 100 ml volumetric flask.

- interferences solutions (1000 ppm) were prepared by dissolving 100 mg of each interferences in exactly 100 ml of deionized water.

2.2. Recommended procedures

2.2.1. Method A

Different volumes of MET ranging from 0.03 to 3.0 ml (0.3-30 µg /ml) were transferred into a series of 10 ml volumetric flasks followed by adding 0.2 ml of 0.3% NBD-Cl and 1.0 ml of 2M NaOH, then completed to the mark with deionized water. After 5 min, the absorbance of the colored product was measured at 546 nm at room temperature against reagent blank.

2.2.2. Method B

Different aliquots of working standard of MET ranging from 0.05 to 4.0 ml (0.5-40 µg /ml) were transferred into a series of 10 ml volumetric flasks. To each flasks, 0.5 ml of 2M sodium carbonate and 0.3 ml of 1:3 FCR reagents were added, and then diluted to the mark with deionized water and kept at room temperature for 10 min to complete the reaction. The absorbance of the blue colored complex was measured at 725 nm against reagent blank.

2.3. Assay procedure for pharmaceutical tablets

Two different brands of metronidazole tablets were analyzed. Twenty tablets for each brand of metronidazole were powdered. An accurate quantity of powdered tablets equivalent to 0.01 g of MET was weighed and dissolved in about10 ml of deionized water, then the procedure of reduction (section 2.3) was applied. The solution was then filtered, and the residue was washed out thoroughly with 10 ml portions of deionized water (three times).The total volume of the filtrate was then made up to 100 ml with deionized water and the recommended procedure was applied for determination of MET. The absorbance of the solution was measured against the solution

blank and the amount of MET was calculated from the calibration graph.

2.4. Assay procedure for suspended solution

0.25 ml of the suspended solution (equivalent to 0.01 g of MET) was diluted with 10 ml of distilled water and the procedure of reduction (section 2.3) was applied. The solution was then diluted to the mark in a 100 ml volumetric flask with deionized water to get the concentration of 100 ppm MET. The recommended procedure was then applied for determination of MET. The absorbance was measured against the blank solution and the amount of MET was calculated from the calibration graph. The readings were taken in triplicate.

3. RESULTS AND DISCUSSION

The proposed spectrophotometric methods for determination of metronidazole were based on the reduction of the nitro group to an amino group by powdered iron in an acidic medium (HCl). The resulting amine undergoes a rapid reaction in alkaline medium with NBD-Cl /NaOH and FCR/Na2CO3 to form two colored complexes with maximum absorbances at 546 and 725 nm, respectively. NBD-Cl and FCR have been used as chromogenic reagents for the colorimetric determination of phenolic compounds and amines (primary and secondary) and for spectrophotometric determination of compounds such as acyclovi certain nitrogen compounds,salbutamol,lisinopril , paroxetine hydrochloride, hydralazine hydrochloride , pencillin, sencnidazole and ferulic acid (Basavaiah and Prammela, 2002; Ikawa et al., 2003; El-Enany et al., 2004; El-Emam et al., 2004; Onal et al., 2005; Siddappa et al., 2009; Singh and Maheshwari, 2010; Kuma et al., 2012; Jadhav et al., 2012). In the method A, the reaction is a nucleophilic Aromatic Substitution (SNAr) in which the chlorine atom of NBD-Cl reagent attacks the primary amino group of the drug in alkaline medium followed by the liberation of hydrochloric acid to form a purple colored complex that shows a maximum absorption at 546 nm (Fig. 2).

In the method B, the estimation is based on reacting of MET with Folin-Ciocaleteu reagent in an alkaline medium to form a blue colored product that shows maximum absorption at 725 nm (Fig. 2). Folin-Ciocaleteu reagent (FCR) is a mixture of phosphomolybdate & phosphotungstate. The complex formation was due to the oxidation of the drug and the reduction of FCR. The proposed reaction mechanisms based on the reported methods (Kuchekar et al., 2005; Swamy et al., 2012) are given in scheme 1.

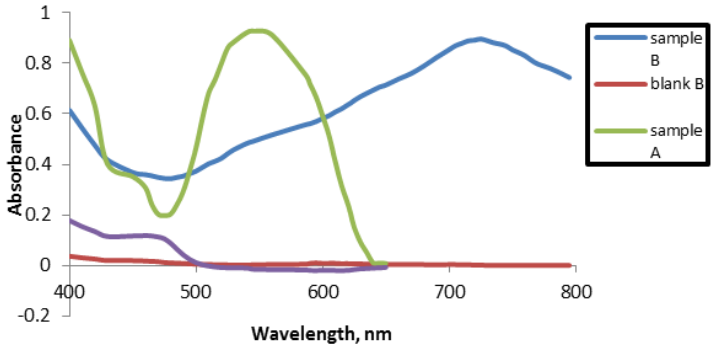
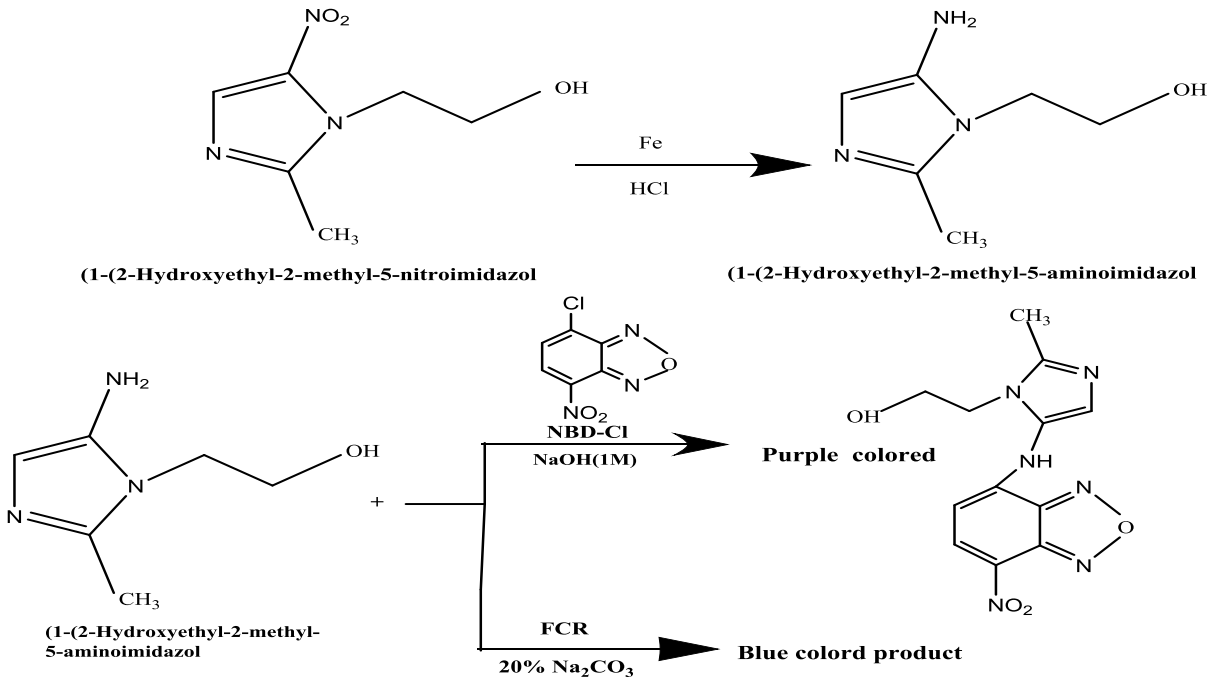


Fig.2. Absorption spectra of MET (20 µg / ml) for methods A and B against reagent blank.



Scheme 1. Proposed chemical reactions of MET in basic medium with NBD-Cl and FCR

3.1. Optimization of reaction variables

3.1.1. Effect of reagents

The influence of the concentration of NBD-Cl and FCR was studied using different volumes of 0.3% NBD-Cl or 1:3 diluted FCR solutions of the reagent on the intensity of the developed color at constant MET concentration (20 µg/ ml). It was found that 0.2 ml of NBD-Cl and 0.3 ml of FCR give the maximum absorbance; while above this volume the absorbance decreases (method B) or remains constant (method A) (Fig.3). Therefore 0.2 ml of (NBD-Cl) and 0.3 ml of (FCR) were selected as the optimum volumes and used in all subsequent measurements.

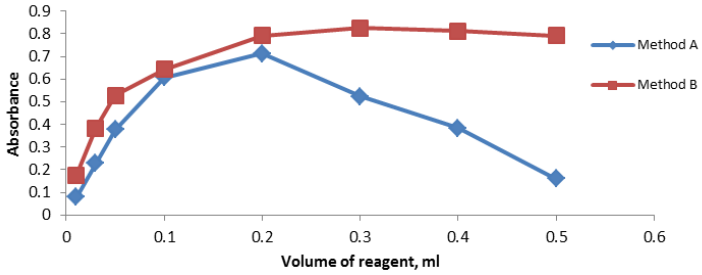


Fig. 3. Effect of volume of 0.3% NBD-Cl and 1:3 diluted FCR on the absorbance of the final colored product in the solution of MET (20 µg/ ml).

3.1.2. Effect of alkaline solution

To obtain the maximum absorbance ,the effect of concentration of different types of alkaline solution were studied .Different volumes ranged from 0.3-2.5 ml of 2M of three base solutions (sodium hydroxide, potassium hydroxide, and sodium bicarbonate) were examined. The results showed that the maximum absorbances can be obtained by

using 1 ml of 2M sodium hydroxide in method A and 0.5 ml of 2M sodium carbonate in method B (Fig. 4). Hence, these volumes were selected and used in all subsequent measurements.

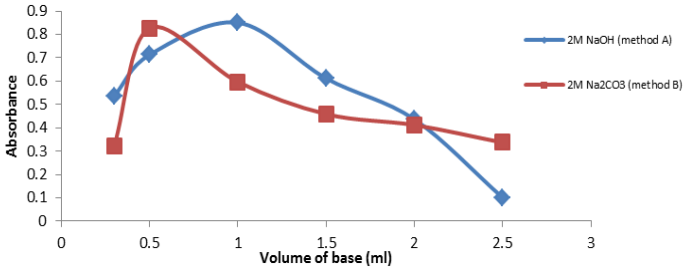


Fig. 4. Effect of different concentrations of alkaline solutions (NaOH and Na_2CO_3) on the absorbance of the final reaction solution.

3.1.3. Effect of temperature and time of reaction

It was found that an increasing of the temperature has a negative effect on the absorbance of the reaction solution for both methods A and B. the reason may be due to the degradation of the main produced colored product in the reaction .The effect of temperature ranged from 20-60°C and reaction times on the color intensity were studied. It was found that an increasing of the temperature has a negative effect on the absorbance of the reaction solution for both methods A and B.The reason may probably due to the degradation or instability of the main produced colored product in the reaction solution. As shown in Table 1,the maximum color development was obtained at room temperature (25°C) (optimum temperature) after 5 min and 10 min for methods A and B ,respectively and they were used throughout the determination process for method A and B ,respectively (Table 1).Afterwards, the stability of the product in both method A and B were monitored by measuring the absorbance of the final colored solution. It was found that the final colored products are stable for up to 1 hour (method A) and 3 hours (method B) at 25°C as shown in Fig. 5.

Table 1
Effect of temperature and time on the absorbance of final reaction solution.

Temp.° C	Time (min) with the absorbance values (method A)					Time (min) with the absorbance values (method B)				
	5	10	20	30	40	5	10	20	30	40
20	0.872	0.869	0.867	0.866	0.864	0.887	0.886	0.883	0.880	0.878
25	**0.915**	0.910	0.908	0.909	0.906	0.890	**0.892**	0.889	0.884	0.885
30	0.856	0.854	0.852	0.848	0.849	0.829	0.833	0.830	0.829	0.827
40	0.811	0.809	0.807	0.806	0.803	0.784	0.787	0.785	0.783	0.781
50	0.673	0.671	0.670	0.669	0.666	0.711	0.714	0.712	0.710	0.710
60	0.522	0.521	0.519	0.517	0.515	0.685	0.689	0.887	0.885	0.883

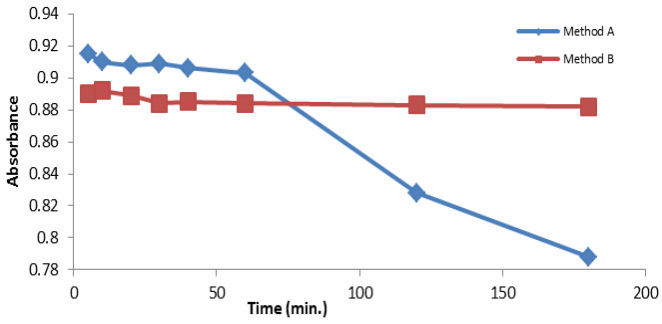


Fig. 5. Effect of time on the stability of the final colored products in methods A and B.

3.1.4. Effect of order of addition

The order of addition of reagent (R) and corresponding volume of base (B) to the sample solution (20 ppm of MET) was examined (Table 2). The results indicated that the addition orders (II) in method A and (I) in method B are the optimum as they produced the colored products with high intensity.

Table 2
Effect of order of addition on the absorbances.

Reaction components	Order number	Absorbance (method A)	Absorbance (method B)
MET + B+ R	I	0.745	0.896
MET + R +B	II	0.916	0.711
R + B +MET	III	0.577	0.448

3.1.5. Effect of solvents

In order to choose the best solvent for color development of the solution, different solvents were tested such as methanol, ethanol, acetone, acetonitrile and water. The results indicated that the highest absorbance and reproducible results are obtained when water is used as a solvent (Table 3). Hence deionized water was selected for dilution of NBD-Cl and FCR solutions throughout all subsequent experiments.

Table 3

Effect of solvents on optical properties of the color products.

Solvents	Absorbance of Method A	Absorbance of Method B
Methanol	0.301	0.287
Ethanol	0.211	0.118
Acetone	Turbid	Turbid
Acetonitrile	Turbid	Turbid
Water	0.917	0.898

3.2. Analytical methods validation

3.2.1. Analytical data

The analytical parameters such as Beer's law limits, Sandell's sensitivity and molar absorptivity were calculated for the proposed methods A and B (Table 4). Regression analysis of the Beer's law also revealed a good correlation.

Table 4

Analytical parameters of methods A and B for determination of MET.

Parameters	The value (Method A)	The value (Method B)
Color	Purple	Blue
Medium	NaOH (2 M)	Na_2CO_3 (2 M)
λ_{max}, nm	546	725
Beer's law range (µg / ml)	0.3-30	0.5-40
LOD (µg / ml)	0.16	0.05
LOQ (µg/ml)	0.56	0.18
ε (l/mole. cm)	0.712×10^4	0.685×10^4
Sandell's sensitivity (µg/cm^2)	0.024	0.025
Regression equation : Y = bX + a*		
Intercept (a)	0.1058	0.103
Slope (b)	0.0416	0.04
Determination coefficient (R^2)	0.9982	0.9967
RSD%	0.225	0.31
Recovery%	100.69	99.04
Stability (h.)	1	3

* Y is absorbance and X is concentration.

3.2.2. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) indicate the sensitivity of the method. LOD is the lowest detectable concentration of the analyte by the method while LOQ is the minimum quantifiable concentration. LOD and LOQ were calculated according to the International Union of Pure and Applied Chemistry (IUPAC) definition (IUPAC, 1978):

$LOD = 3\ \sigma/s$ and $LOQ = 10\sigma/s$

Where: σ is the standard deviation of replicate measurements under the same conditions as used for the sample analysis in the absence of the analyte; s is the sensitivity of the method i.e. the slope of the calibration curve. The results showed in Table 4 indicate the high sensitivity of the proposed methods.

3.2.3. Precision and accuracy

The accuracy and precision of the proposed methods were evaluated by replicate analysis (n =5) of calibration standards at three concentration levels as the RSD and recovery percentage were calculated for both methods A and B. The results illustrated in Table 5 indicate that the methods were satisfactory and have a good accuracy and precision.

Table 5

Accuracy and precision of the proposed methods A & B.

MET taken, µg/ml	MET found, µg/ml for method A	MET found, µg/ml for method B	Recovery, % for method A	Recovery, % for method B	RSD, %* for method A	RSD, %* for method B
5.0	5.08	4.79	101.6	95.8	0.236	0.46
15.0	15.12	15.28	100.8	101.8	0.244	0.24
25.0	24.92	24.88	99.68	99.52	0.195	0.23
	Average		100.69	99.04	0.225	0.31

*Average of five determinations.

3.3. Interference study

The effects of common excipients used in the pharmaceutical preparations on the performing of the proposed analytical methods were studied. It was carried out by analyzing a synthetic sample solution containing 20 µg of MET in the presence of different amounts of excipients (starch, glucose, lactose and sodium chloride).Experimental results showed that the excipients or additives up to 500 µg has no impact on the determination of MET (Table 6).

Table 6
Effect of excipients for assay of MET.

Excipients	µg Excipients	Recovery% (n=3) for method A	Recovery% (n=3) for method B
Starch	100	101.2	101.2
	200	102.8	100.9
	500	96.7	99.2
Glucose	100	100.7	99.8
	200	98.4	101.9
	500	97.6	102.5
Lactose	100	٩9.8	100.8
	200	98.1	101.3
	500	96.9	98.5
Sodium chloride	100	99.3	101.1
	200	98.9	97.8
	500	97.7	102.7

3.4. Application to the analysis of pharmaceutical formulations

The proposed methods were applied successfully for determination of MET in pharmaceutical formulations. Validation of the proposed methods A and B was performed statistically (Miller and Miller, 2005) by taking five replicate measurements and then comparing the obtained results with those corresponding data obtained by the official BP method (British Pharmacopeia, 2013). No significant difference was found by applying t-test and F-test at 99% confidence level with four degrees of freedom (Table 7). The results showed that the experimental t-test and F-test were less than the theoretical value (t=3.747, F=15.97)(Table 7) and indicating that there was no significant difference between the results obtained by the proposed methods and standard method.

Table 7
Applications of methods A & B for the determination of MET and statistical comparison with the official method.

Pharmaceutical preparations of MET	% Found [1] ±SD[2]		
	Proposed methods		Official method
	Method A, t[3] & F[4] values	Method B, t[3] & F[4] values	
Nidazole 200 mg (suspension), HIKMA Pharma. Jordan	97.2± 0.53 t=1.92 F=1.00	97.0±0.66 t=1.80 F=4.79	96.4±1.14
Metronidazole Awa 500 mg (tablet), Awamedica, Erbil, Iraq	101.6±0.82 t=1.98 F=٠.59	97.6±0.77 t=1.91 F=0.98	97.0±0.46
Flanizol 500 mg (tablet), United Pharma. Jordan	98.4±0.66 t=1.91 F=٠.85	95.4±0.97 t=1.98 F=1.48	99.2±0.16

[1] Average of five determinations; [2] SD=Standard deviation; [3] Tabulated t-value for four degrees of freedom; and p=0.01 is 3.747; [4] Tabulated F-value for four degrees of freedom; and p=0.01 is 15.97.

3.5. Comparison of the methods

Several analytical variables were taken into account to make a comparison between the developed methods and other previously reported spectrophotometric methods (Table 8).It indicates that the developed methods are sensitive due to the high values of molar absorptivity enough stability of the colored products.

Table 8
Comparison between the proposed methods and previously reported methods for determination of MET.

Analytical parameters	Proposed methods		Previouslyreported method *	Previously reported method **
	Method A	Method B		
Color	Purple	Blue	Pinkish-red	Purple
Medium	Aqueous (2M NaOH)	Aqueous (2M Na_2CO_3)	Aqueous (3% sulphamic acid)	1, 4-dioxan/Acetonitrile (6:4) mixture
Type of reaction	Coupling reaction	Coupling reaction	Diazo-coupling reaction	charge-transfer complexation
Reagent	NBD-Cl	FCR	N-(1-naphthyl) ethylenediamine	chloranilic acid
Temperature (°C)	25	25	At room temperature	At room temperature
λ_{max} , nm	546	725	503	520
Beer's law range (µg/ml)	0.3-30	0.5-40	0.8-20	5-40
Determination coefficient, R^2	0.9982	0.9967	0.9999	0.9990
ε (l/mol.cm)	0.712×10^4	0.685×10^4	4.673×10^3	1.312×10^3
LOD (µg/ ml)	0.16	0.05	……..	……..
RSD%	0.225	0.31	±0.47 to ±1.08	……..
Stability of the color	1 h	3 h	75 minutes	…….
Applications	Suspension and tablets	Suspension and tablets	Suspension ,tablets and injection	Dosage forms

* Ibrahim and Bashir, 2012.
** Adegoke, 2011.

4. CONCLUSIONS

The important features of the developed methods compared to other previously used techniques for the determination of metronidazole are sensitivity, easy accessibility to the instrument and cost –effectiveness. The reagents used in the proposed methods are safe, cheap and readily available. The statistical analysis showed that the data from the proposed methods are in a good agreement with those reported in literature. The high values of molar absorptivity, low values of Sandell's sensitivity and LOD indicate the high sensitivity of the proposed methods. The methods are free from critical optimum conditions such as heating, extraction step, pH control and using of toxic organic solvents. The colored products obtained from the reaction were enough stable to be analyzed by the analyst. Moreover, the developed methods are not affected by interferences such as excipients or additives. The presented data in this paper for determination of metronidazole in its pure and dosage forms demonstrate that the proposed methods have an acceptable linearity, high accuracy and precisio

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