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Influence of Some Physicochemical Exposure Factors on the Metronidazole Content of a Pharmaceutical Product: Flagyl® 250 mg Tablet

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Abstract

The present work aims to study the influence of some physicochemical parameters (light, temperature, ethanol, bile salts, potassium hydroxide, hydrogen peroxide) on the content of Metronidazole (MTZ) contained in the reference substance (SR) and in a pharmaceutical specialty Flagyl® 250 mg tablet (FLG). The method developed was linear and accurate in accordance with USP 38 and the MTZ contents were obtained by UV-visible spectrophotometry at 278 nm. These contents ranged from 225 mg to 275 mg and were thus consistent with the concentration present in the proprietary medicines (250 mg). The study of the influence of physicochemical parameters on the MTZ content in SR and FLG showed that MTZ contents are unstable in the presence of Ethanol at 96˚, KOH at 0.1N and bile salts but also when the temperature is higher than 25˚C. However, they remain stable in the presence of light and H_2O_2 and undergo degradation in an acidic environment.

Keywords

Metronidazole, Influence, Physicochemistry, Content, Stability

1. Introduction

The stability study of drugs is an important step in a pharmaceutical industry. It is a requirement for obtaining marketing authorization for the drug and for maintaining the renewal of this authorization. It is the guarantee of the quality of

the drug and the safety of its use [1]. The stability study provides evidence of how the quality of a product changes with time and under various environmental factors, while establishing storage conditions and duration. The stability study provides evidence of how the quality of a product varies over time and under various environmental factors, while also establishing storage conditions and duration. The conduct of a stability study program should take into account the target market as well as the climatic conditions of the region in which the drugs will be marketed. This study must be conducted on all sizes and types of packaging planned for the marketing of the product [2]. The WHO believes that it is important to set strict standards for medicines to ensure their stability over time [3]. In developing countries, particularly in sub-Saharan Africa, the magnitude of the stability problem is considerable due to the tropical climate, storage conditions, and the fate of the drug once consumed [4] [5]. Antibiotics and antiparasitics are not left out of this stability study [6] [7]. Among these is Metronidazole, an antibiotic and antiparasitic belonging to the nitroimidazoles. It inhibits nucleic acid synthesis and is used for the treatment of infections related to anaerobic bacteria as well as protozoa. A search for a treatment against *Trichomonas vaginalis* led to the first clinical trials in 1959 of metronidazole against this parasite. Researchers in France show the effectiveness of this molecule against the parasite, continuing in 1960 with positive studies in the United Kingdom confirming its use against Trichomonas [8].

In Ivory Coast, metronidazole is a much sought-after molecule, either alone or in combination, in the management of various pathologies in both children and adults. These include giardiasis and intestinal amoebiasis [9], amebic liver abscesses [10] and gastric and duodenal ulcers involving Helicobacter pylori [11]. In a 2014 study by Kouassi and colleagues [12] on antimicrobial susceptibility to *Clostridium perfringens* and *Clostridium difficile*, metronidazole and vancomycin were found to be most active on *C. difficile*, while metronidazole and Penicillin G were most active on *C. perfringens*.

The purpose of this study is to evaluate the stability of metronidazole under various physical and chemical conditions such as light, temperature, ethanol, bile salts, potassium hydroxide and hydrogen peroxide.

2. Materials and Methods

2.1. Sampling

Flagyl® 250 mg, lot number OR1L7, from a pharmacy in Abidjan (Ivory Coast), was used as a sample for the study. The choice of this pharmaceutical speciality is explained by the fact that it is the original speciality that obtained the first marketing authorisation.

2.2. Reference Substance and Chemicals

Metronidazole of 99% purity at 250 mg from the German manufacturer GPHF (Global Pharma Heath Fund), lot number 5MJ175, was used as the reference

substance.

All chemicals used were of analytical quality: hydrochloric acid (HCl) (37%, CARLO ERBA, France), potassium hydroxide (KOH (1000 $mg{\cdot}L^{-1}$, CARLO ERBA, France), ethanol (C_2H_5OH) (96%, CARLO EBRA, France), hydrogen peroxide (H_2O_2) (10 volumes, GILBERT, France), bile salts (FLUKA analytical, France).

Distilled water was used for the preparation of the reference substance and the various reagents.

2.3. Equipment

A METTLER TOLEDO analytical balance with a precision of 0.0001 g was used for weighing chemical masses. A Specord 210 plus double beam UV-visible spectrophotometer (Analytik Jena, France) equipped with a combined halogen and deuterium lamp, a tempered detector system and a monochromator was used for the determination of Metronidazole content. The pH of the solutions was read using the Lovibond pH meter brand SD 305 pH/ORP calibrated at pH 4.01, 7.0 and 10.0. The experiments also required a VWR ultrasonic bath (15 - 400 kHz).

2.4. Methods

2.4.1. Preparation of the Reference Substance Standard Range (SR)

A mass of 10 mg of SR was weighed using the analytical balance and dissolved in 5 mL of 0.1 N HCI in a 100 mL volumetric flask. The contents of the flask were then homogenized with the ultrasonic bath for 15 min and the flask was made up to the mark with 0.1 N HCI to obtain a stock solution of concentration 100 $\mu g{\cdot}mL^{-1}$. From this stock solution obtained, daughter solutions were prepared at concentrations of 2 $\mu g{\cdot}mL^{-1}$, 4 $\mu g{\cdot}mL^{-1}$, 6 $\mu g{\cdot}mL^{-1}$, 8 $\mu g{\cdot}mL^{-1}$, 10 $\mu g{\cdot}mL^{-1}$ and 12 $\mu g{\cdot}mL^{-1}$ respectively. Each daughter solution was read by UV-visible spectrophotometer, taking three readings for each concentration (Table 1).

2.4.2. Preparation of Sample Solutions

A quantity of twenty tablets was weighed using the analytical balance and the average corresponding to the twenty weighed tablets was calculated. Then, the tablets were ground to fine powder in a mortar, and the equivalent of 10 mg of

Table 1. Preparation of the standard range of the reference substance.

Vials used	SR1	SR2	SR3	SR4	SR5	SR6
Concentration of Stock Solution SM ($\mu g{\cdot}mL^{-1}$)	100	100	100	100	100	100
Volume of SM collected (µL)	400	800	1200	1600	2000	2400
Volume of HCI used to adjust the via (mL)	19.60	19.20	18.80	18.40	18.00	17.60
Volume of flask used for dilution (mL)	20	20	20	20	20	20
Concentration of daughter solutions obtained ($\mu g{\cdot}mL^{-1}$)	2	4	6	8	10	12

powder was weighed and transferred to a 100 mL flask containing 5 mL of 0.1 N HCI to obtain a concentrated stock solution of 100 $\mu g \cdot mL^{-1}$. Daughter solutions with concentrations of 2 $\mu g \cdot mL^{-1}$ (SE1), 6 $\mu g \cdot mL^{-1}$ (SE3) and 10 $\mu g \cdot mL^{-1}$ (SE5), respectively, were obtained from successive dilutions of the stock solution (Table 1). Absorbance readings were taken with a UV-visible spectrophotometer, taking three readings for each solution.

2.4.3. Preparation of Working Solvents

A 0.1 N KOH solution was obtained by dissolving 56.10 mg of KOH powder in 10 mL of distilled water.

The 100 $\mu g \cdot mL^{-1}$ bile salt solution was prepared by dissolving 10 mg of bile salts in distilled water in a 100 mL volumetric flask.

The other solvents, namely Ethanol at 96˚ and hydrogen peroxide (H_2O_2) at 10 volumes, were used without being diluted.

2.4.4. Determination of Validation Parameters

These parameters are linearity, precision, accuracy and limits of detection and quantification.

- **Linearity**

Linearity was assessed by preparing a standard range of 2 $\mu g \cdot mL^{-1}$ to 12 $\mu g \cdot mL^{-1}$ by successive dilutions of the reference solution (SR). The calibration curve was established from the absorbances obtained for each of the concentrations, and the coefficient of determination (R^2) and the equation of the line were deduced. However, prior to the linearity determination, a spectral scan (from 200 to 600 nm) was performed to determine the maximum absorption wavelength λ_{max} (Figure 1) and plot the calibration curve (Figure 2).

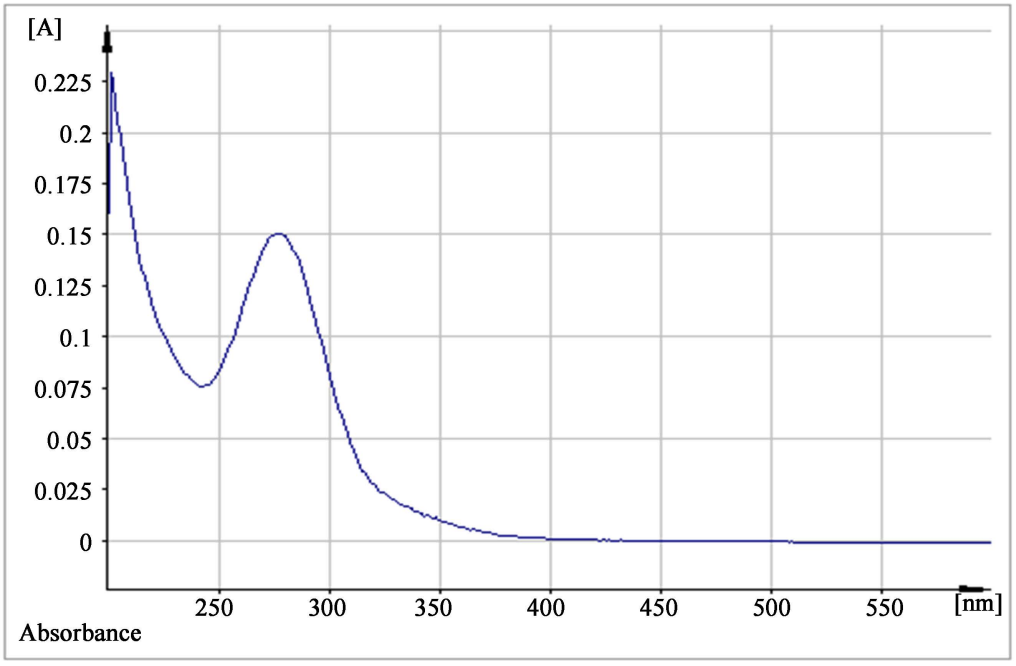

Figure 1. Absorption spectrum of MTZ daughter solution at 6 µg/mL.

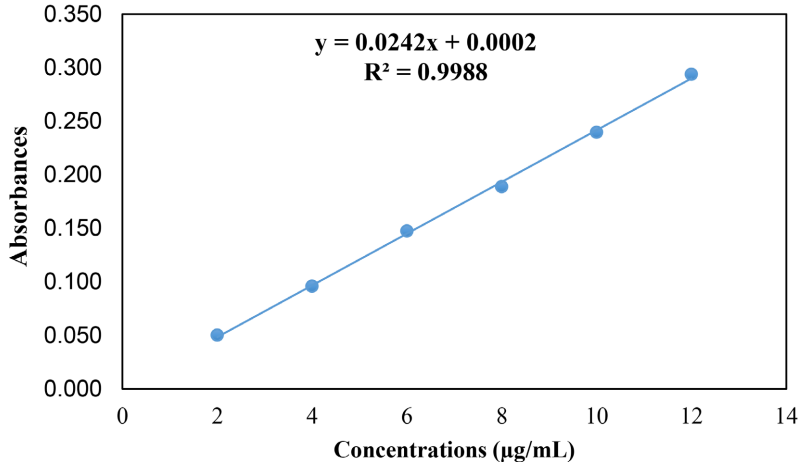

Figure 2. Calibration curve of the MTZ standard solution at 278 nm.

- **Loyalty**

Intra-day precision or repeatability was determined by taking eighteen readings (*i.e.*, three sets of six successive readings) of the SR3 daughter standard (C = 6 µg·mL^{-1}) on the spectrophotometer on the same day with a four-hour delay.

Intermediate or inter-day fidelity was determined by taking eighteen readings (*i.e.*, three sets of six successive readings) of the SR3 daughter standard (C = 6 µg·mL^{-1}) in the spectrophotometer on three consecutive days.

The mean absorbance of the three sets of readings and the coefficients of variation (CV) were calculated for each test and the data reported in Table 2.

- **Accuracy**

The accuracy test was performed by adding a 5 mL volume of the reference solutions SR1 (C = 2 µg·mL^{-1}, q = 10 µg), SR3 (C = µg·mL^{-1}, q = 30 µg) and SR5 (C = 10 µg·mL^{-1}, q = 50 µg) to a 5 mL volume of sample (*i.e.*, 30 µg of the sample solution SE3 = 6 µg·mL^{-1}). The absorbances obtained after reading were converted to µg/mL using the equation line and then to µg (by multiplying the concentration obtained from the equation line by the total volume of the different mixtures).

The recovery percentages (RP) were calculated according to the following formula (Equation (1)) and listed in Table 3:

$$PR = \frac{QF - QI}{QA} \times 100 \quad (1)$$

where PR is the Recovery Percentage; QF, QI and QA are the Fortified, Initial and Added Quantities respectively.

The Fortified Quantities (FQ) are determined from the obtained absorbance and the equation line.

- **Limit of detection and Limit of quantification**

They were assessed from successive dilutions of 1/2, 1/4, 1/8, 1/200 and 1/400 of the MTZ reference substance at 2 µg·mL^{-1} (Table 4).

2.4.5. Determination of MTZ content

The pharmaceutical product used in the study was assayed as follows (Equation (2)):

Table 2. Intra-day and inter-day reliability of the method.

Concentrations (µg/mL)	Loyalty Intra-day 8 hours		
	Average Absorbance	Mean ± Standard deviation	CV (%)
6	0.148		
6	0.148		
6	0.148	0.148 ± 0.001	0.371
6	0.147		
6	0.147		
6	0.147		
Loyalty Intra-day 12 hours			
6	0.148		
6	0.147		
6	0.147	0.147 ± 0.001	0.528
6	0.149		
6	0.147		
6	0.146		
Loyalty Intra-day 16 hours			
6	0.149		
6	0.150		
6	0.148	0.096 ± 0.002	0.522
6	0.148		
6	0.149		
6	0,150		

Amount SR (µg/mL)	Inter-day loyalty Average absorbance		
	1er Day	2ème Day	3ème Day
6	0.146	0.141	0.150
6	0.147	0.140	0.150
6	0.147	0.141	0.151
6	0.147	0.140	0.150
6	0.146	0.141	0.150
6	0.147	0.141	0.150
Average	0.147	0.141	0.150
Standard deviation	0.001	0.001	0.001
CV (%)	**0.680**	**0.709**	**0.666**

Table 3. Accuracy of the method.

Quantity Sample (µg)	Amount of SR added (µg)	Total amount recovered (µg)	Recovery rate (%)
30	10	39.870	98.698
30	30	60.237	100.791
30	50	79.546	99.091
Recovery rate (%)			99.527
Standard deviation			0.843
CV (%)			0.847

Table 4. Limit of detection and limit of quantification of the method.

Dilution factor	Concentration (µg/mL)	Absorbance
1/2	1	0.118
1/4	0.5	0.025
1/8	0.25	0.012
1/200	0.01	------
1/400	0.005	------

$$\text{Teneur(FLG en mg)} = \frac{\text{Tx recouv}}{100} \times \text{Teneur}\left(\text{FLG conditionnement}\right) \quad (2)$$

with

$$\text{Tx recouv} = \frac{\text{Qte obetnue}}{\text{Qte prise}} \times 100 \quad (3)$$

The data for the determination of the MTZ content are summarized in the Table (**Table 5**).

2.4.6. Influence of Physico-Chemical Parameters on MTZ Content

- **Influence of physical parameters**

The influence of temperature was studied by preparing three paired solutions of SR and FLG at 6 µg·mL^{-1} and subjecting them to different temperatures (25˚C, 37˚C and 50˚C) for 10 minutes. Then, the absorbance versus temperature curve was plotted (**Figure 3**). The previously prepared pairwise solutions were exposed to the laboratory light for three successive days. The absorbances were measured each day after exposure to light and noted. The values obtained were used to plot the influence of light on the MTZ content of SR and FLG as a function of time (**Figure 4**).

- **Influence of chemical parameters**

The study of the influence of ethanol at 96˚ was carried out by adding respective solutions of SR and FLG of concentration 6 µg·mL^{-1} to 1 mL of Ethanol at 96˚ contained in two separate volumetric flasks of 20 mL. The solutions obtained were kept protected from light for three successive days, the absorbances

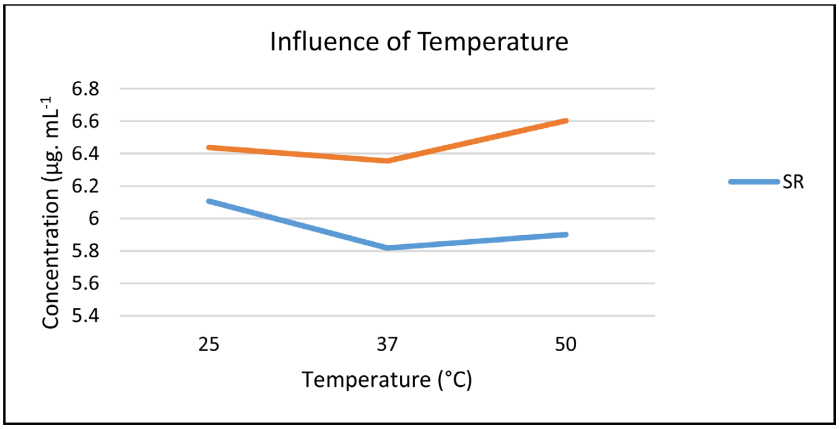

Figure 3. Temperature evolution curve on MTZ content in SR and FLG.

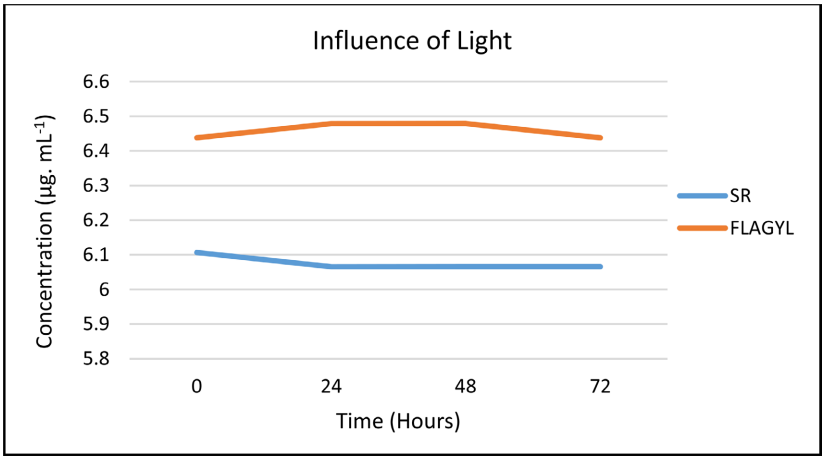

Figure 4. Evolution of the influence of light on the content of MTZ contained in SR and FLG.

corresponding to each day were measured and the curve of the influence of 96˚ Ethanol on the MTZ content of SR and FLG as a function of time was plotted (**Figure 5**).

The influence of bile salts was studied by adding respective solutions of SR and FLG of concentration 6 µg·mL^{-1} to 1 mL of bile salts contained in two separate 20 mL volumetric flasks. The solutions were kept in the dark for three weeks. The absorbances were then measured for each week and the time course of the influence of the bile salts on the MTZ content of the SR and FLG was plotted (**Figure 6**).

The influence study was carried out by adding respective solutions of SR and FLG of concentration 6 µg/mL to a volume of 1 mL of 0.1 N KOH contained in two separate 20 mL volumetric flasks. After keeping these solutions protected from light for three weeks, the relative absorbances for each week were measured and the curve of the influence of KOH on the content of MTZ contained in SR and FLG was plotted (**Figure 7**).

To study the influence of hydrogen peroxide (H_2O_2), respective solutions of SR and FLG dosed at 6 µg/mL were added to 1 mL of H_2O_2 contained in two

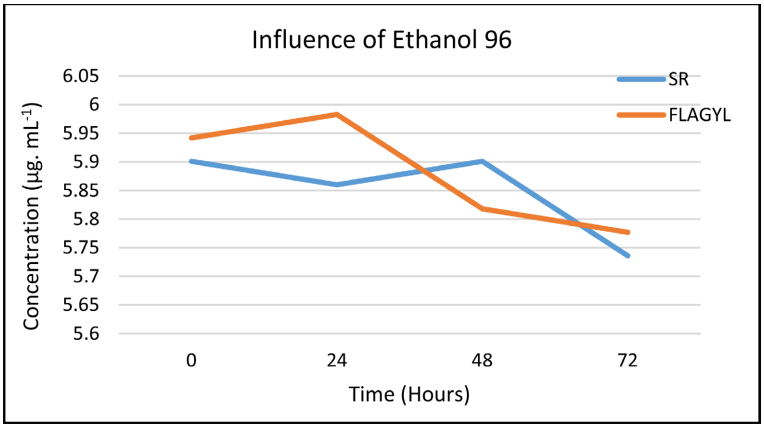

Figure 5. Evolution curve of Ethanol at 96˚ on the content of MTZ contained in SR and FLG.

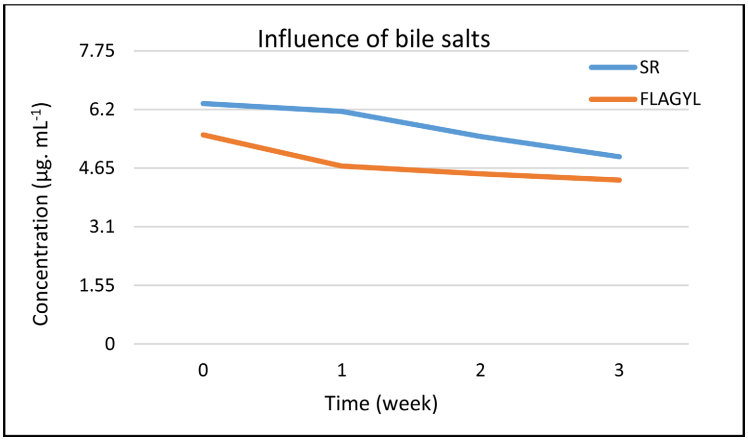

Figure 6. Evolution curve of bile salt on MTZ content in SR and FLG.

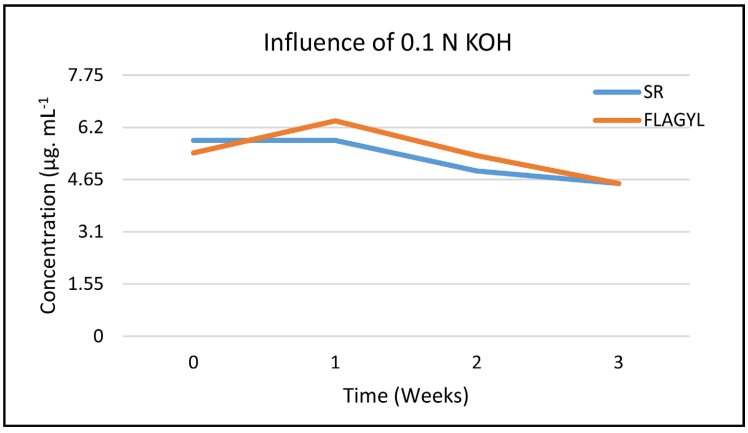

Figure 7. Evolution curve of KOH on MTZ content in SR and FLG.

separate 20 mL volumetric flasks. The resulting solutions were protected from light for three successive days. Then the absorbances corresponding to each day were read and the evolution curve of the influence of H_2O_2 on the content of MTZ contained in SR and FLG was plotted (**Figure 8**).

The study of the influence of pH consisted in varying the pH of the respective

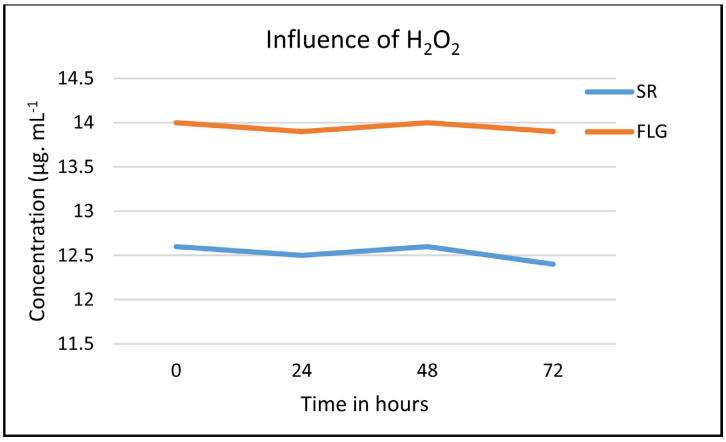

Figure 8. H_2O_2 evolution curve on the MTZ content in SR and FLG.

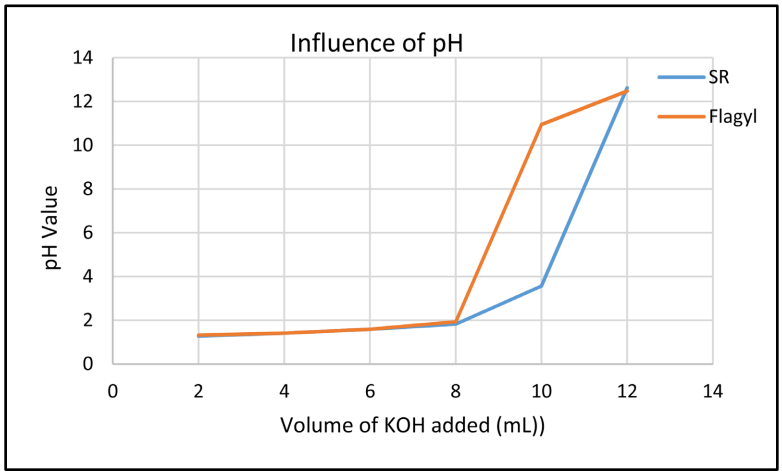

Figure 9. Evolution curve of pH versus KOH volume.

solutions of SR and FLG of concentration 6 µg/mL (of acidic pH). Thus, respective volumes of 2.4, 6.8, 10 and 12 mL of a basic 1 N KOH solution were added to these solutions contained in two separate 100 mL volumetric flasks. A contact time of one hour was observed for these different solutions obtained. After this contact time, their respective pH values were determined. The evolution curve of the pH determined as a function of the volume of KOH poured in was plotted (**Figure 9**), then the absorbances corresponding to each solution were measured and the evolution curve of the influence of pH on the content of MTZ contained in SR and FLG as a function of time was plotted (**Figure 10** and **Figure 11**).

2.4.7. Data Processing

MICROSOFT EXCEL version 2016 software was used for data processing.

3. Results and Discussion

3.1. Validation of the MTZ Assay Method

Before the study of the validation parameters of MTZ, we proceeded to determine the absorption spectrum of MTZ by performing a spectral scan in the

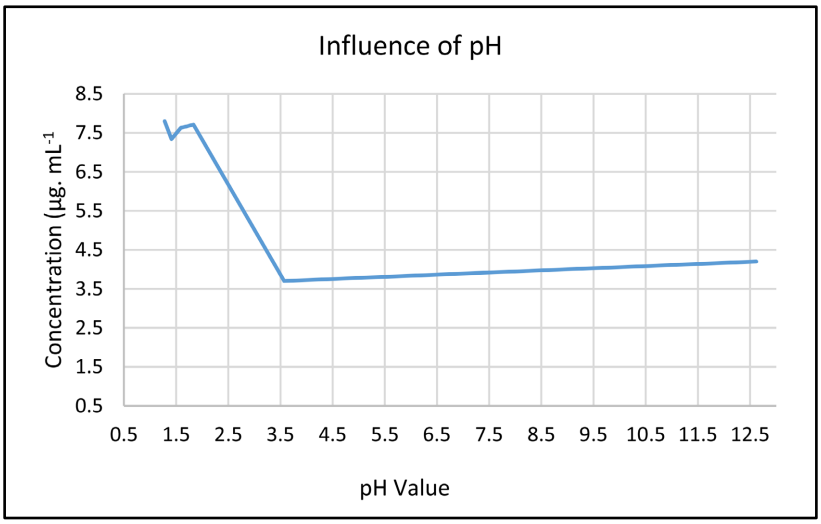

Figure 10. Evolution curve of pH on MTZ content in SR.

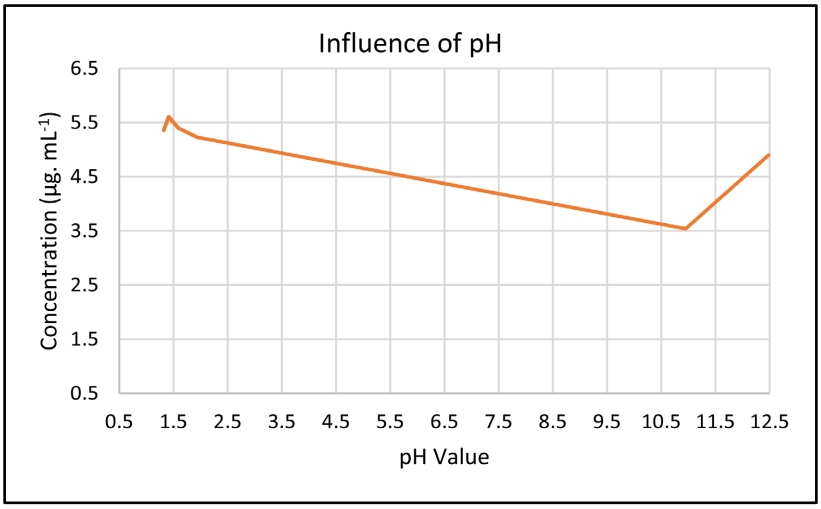

Figure 11. Evolution curve of pH on MTZ content in FLG.

UV-visible range from 200 nm to 600 nm. This spectral scan detected the maximum wavelength λ_{max} of MTZ which is 278 nm as shown in **Figure 1**.

The spectral data of the standard solution was used to plot the calibration curve of MTZ at 278 nm (**Figure 2**).

The linearity of the method was determined by assessing the coefficient of determination R^2 obtained from the calibration curve. This coefficient was 0.9988. Our result is in accordance with USP Pharmacopoeia 38 NF 33 version 2015 [13] which states that the coefficient of determination should be greater than 0.9950. Our method is therefore linear.

Also our result is close to those obtained by Das J. and *et al.* [14] and Mastanamma S and et *al.* [15] who found respective values of R^2 of 0.9974 and 0.9994 also in accordance with that of USP Pharmacopeia 38 NF 33 version 2015.

The fidelity of our method gave CVs of 0.371%, 0.528% and 0.522% at 8, 12 and 16 hours for intra-day fidelity and 0.680%, 0.709% and 0.666% for inter-day

fidelity respectively. Our method complies with the CVs given by USP Pharmacopoeia 38 NF 33 version 2015 [13] which states that the CVs for intra-day fidelity and inter-day fidelity should be less than 1% and 1.5% respectively. Therefore our method is faithful.

The accuracy gave an average recovery rate of 99.527% in line with the specifications given by USP Pharmacopoeia 38 NF 33 version 2015 [13] which recommends that the average recovery rate should be contained within the acceptability range of 98% to 102%. Therefore, our method is accurate.

Our result is also close to the one obtained by Sadek S.A. and *et al.* [16] who obtained an average recovery rate of 98.800%.

The limit of detection and limit of quantification of our method were 0.01 µg/mL and 0.25 µg/mL respectively. Our results differ from those obtained by Mastanamma S and *et al.* [15] and Sadek SA and *et al.* [16] in their study who found limits of detection and quantification of 0.15 $\mu g \cdot mL^{-1}$ and 0.46 $\mu g \cdot mL^{-1}$ and 0.4277 $\mu g \cdot mL^{-1}$ and 1.2961 $\mu g \cdot mL^{-1}$ respectively for the first and second authors. This difference could be explained by the fact that they used the calculation method in their study for accuracy assessment.

3.2. Determination of MTZ Content in FLG

The MTZ content in FLG 250 mg is given in **Table 5** below:

The MTZ content in FLG 250 mg was 98.913%. Our result is in accordance with the specifications given by USP Pharmacopoeia 38 NF 33 version 2015 [13] related to sample assay which states that the content of active ingredient should be between 90% and 110% of the amount mentioned on the secondary packaging of the product, *i.e.* 225 mg to 275 mg in our case. The average amount of MTZ contained in FLAGYL 250 mg was 247.282 mg, a value within the range of 225 mg to 275 mg.

3.3. Study of the Influence of Physico-Chemical Parameters on the MTZ Content

Temperature causes variations in MTZ content in SR and FLG above 25˚C. Our result is consistent with that given by the Summary of Product Characteristics (SPC) of MTZ [17] which states that MTZ should be stored at 25˚C and differs from that obtained by Arun K. M and *et al.* [18] who showed in their study that the content of MTZ Benzoate remains stable at 50˚C and degrades from 60˚C.

Table 5. MTZ content in our 250 mg FLG sample.

Specialty. Pharmaceutical	Sample amount taken ($\mu g \cdot mL^{-1}$)	Absorbance	Amount of sample obtained ($\mu g \cdot mL^{-1}$)	Average recovery rate (%)	Average PA amount (mg)
FLG 250 mg	6	0.146	6.025	98.913	247.282
	6	0.147	6.066		
	6	0.148	6.107		

Such a difference could be explained by the fact that their study was done on MTZ Benzoate salt.

When SR and FLG are subjected to the effect of light for 72 h, stability of MTZ content in our SR and FLG is observed. Our result is different from that obtained by Ebeed F. M. A. and *et al.* [19] who showed in their study that the content of MTZ and MTZ Benzoate degrades under the effect of sunlight within 3 h. This difference could be explained by the fact that our compounds (MTZ and FLG) were exposed to laboratory light and room temperature while in the study of Ebeed and *et al.*, the exposure was to sunlight.

The variations observed in the MTZ content after the addition of Ethanol at 96˚ reflect the instability of MTZ in the presence of Ethanol at 96˚. Ethanol at 96˚ therefore degrades MTZ. This degradation could be explained by the fact that MTZ leads to an accumulation of toxic Ethanol molecules (metabolites) in the SR and FLG solutions, thus reducing the MTZ content in these solutions.

The addition of bile salts leads to a decrease in MTZ content in SR and FLG after three weeks. Van Bambeke F. and *et al.* [20] also noted that the intake of MTZ during the meal delays its bioavailability by 100% and this after 1 to 2 hours contrary to our study in which the time was three weeks. We could suppose that the contact of bile salts and MTZ leads to an instability of MTZ, which would be at the origin of the decrease of the content observed in our study. Thus, it would be preferable to administer MTZ away from meals to reduce the impact of bile salts on this compound.

When SR and FLG solutions are put in the presence of 0.1 N KOH for three weeks, degradation of MTZ content in SR and FLG is observed. Our result is in agreement with that of Naveed S. and *et al.* [21] who obtained during their study on the degradation of MTZ and some active formulations by UV-visible spectrophotometry, a decrease in MTZ content in FLG and a stability of MTZ content in MTZ Benzoate after subjecting these compounds in the presence of 0.1 N NaOH. This observed stability could be explained by the fact that the second study was done on MTZ benzoate and in the presence of 0.1 N NaOH instead of 0.1 N KOH used in our study.

The addition of H_2O_2 to our solutions does not influence the MTZ content after 72 hours. This stability of MTZ content in the presence of H_2O_2 (an oxidant) could reflect the fact that MTZ is less sensitive to redox reactions.

The addition of KOH solution in our SR and FLG solutions showed an increasing content of the pH of these solutions ranging from 1.28 to 12.48. The curve of the evolution of the pH as a function of the MTZ contents in SR and FLG shows a strong degradation of MTZ in acidic medium, observed by a fall of the contents going respectively from 7.8 to 3.7 µg/mL and from 5.36 to 3.54 µg/mL for SR and FLG. Our result is consistent with that of Naveed S. and *et al.* [21] who also showed in their study on the degradation of MTZ and some active formulations by UV-visible spectrophotometry, a strong degradation of MTZ in Flagyl in acidic medium.

4. Conclusions

In this work, a method for the identification of MTZ contained in FLG 250 mg tablet was set up and validated by UV-visible spectrophotometry through the validation of parameters such as: linearity, precision, accuracy, limit of detection and limits of quantification. It was found that the MTZ assay was a linear, precise and accurate method with satisfactory validation criteria and could be used for routine analysis for MTZ identification. The MTZ content in the 250 mg FLG was 247.282 mg and the influence of some physicochemical parameters on this content showed its instability in the presence of temperature above 25˚C, ethanol at 96˚, bile salts and KOH at 0.1 N but its stability in the presence of light and H_2O_2 and its degradation in acid environment.

This method should be extended to other types of antiparasitic drugs with a more extensive study of influence on other physicochemical parameters.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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