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A SIMPLE, QUICK, REPRODUCIBLE AND LOW-COST HIGH PERFORMANCE LIQUID CHRO1MATOGRAPHIC METHOD FOR THE ANALYSIS OF METRONIDAZOLE IN TABLETS

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ABSTRACT: A simple, rapid, reproducible and low cost High Performance Liquid Chromatography (HPLC) method was developed and used to assess the quality of some brands of metronidazole tablets being marketed in Abuja, Nigeria. Parameters such as assay for the content of active ingredient, dissolution, disintegration, hardness, friability, and microbial load were evaluated using the methods described in the British Pharmacopoeia. Our results show that, a good linear relationship between Peak Area Response and Concentration in the range of 1.5–50µg/mL and a regression coefficient value of 999 was obtained. The method was found to be very sensitive with values of 25.0 and 15.6ng/mL obtained as Limits of Quantification (LOQ) and Detection (LOD) respectively. The result also showed that all but one (T_5) of the eight brands evaluated met the British Pharmacopeia (BP) specifications for weight uniformity test, disintegration test and dissolution test. The brand T_5, which failed the weight variation test, equally failed the dissolution test. This product was found to have released more than 110 % of the drug content within 45 minutes against the specification in the pharmacopoeia. This product therefore does not comply with the BP dissolution tolerance limits. However, all the brands examined pass the assay for content of active ingredient.

INTRODUCTION: Metronidazole [2-methyl-5-nitroimidazole-1-ethanol] is white or creamy–white crystalline powder with a slight odour, bitter slightly saline taste and it darkens on exposure to light. It is practically soluble in water; alcohol, chloroform and slightly soluble in ether [1]. Metronidazole is one of the azole group antimicrobial agents (**Fig. 1**). It has been evaluated in the treatment of diverse anaerobic and the GIT tract infections.

QUICK RESPONSE CODE

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Moreover, metronidazole has often been studied for antibacterial activity against gram-negative aerobes and some gram-positive bacteria, including *Bacteriodesfragilis* that produces β-lactamases [1]. In healthy humans, metronidazole is absorbed rapidly and completely from the gastrointestinal (GIT) tract and is metabolized in the liver by an oxidative pathway [2]. It is readily absorbed from Gastro-intestinal tract and widely distributed in body tissues. Liver is the main site of metabolism by side chain oxidation and glucuronide conjugation. A major portion of the dose of the drug is excreted in urine, largely as metabolites [3].

Metronidazole is on the Essential Drug List (EDL) recommended by the World Health Organization (WHO). It has antiprotozoal action, effective

against *Trichomonas vaginalis* and some other protozoans that cause infections.

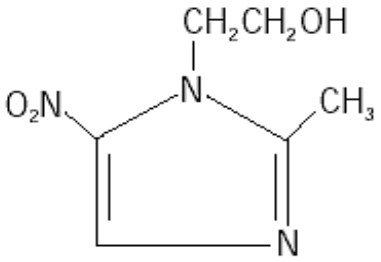

FIG.1: STRUCTURE OF METRONIDAZOLE

In elderly women the metronidazole used in combination with hormonal therapy to clear vaginitis. Metronidazole is used for eradication of cysts in symptomless carries, with 400 to 800mg three times daily for 5 to 10 days [4]. It is often bought as an Over the Counter (OTC) drug, for treatment of stomach upset or loose stools.

The influx of fake, counterfeit and substandard drugs in the Nigerian market is a cause for alarm. The label claims of some of these drugs may not tally with actual content present or maximum differences allowed by official books. This may lead to treatment failure, resistance and even toxicity.

According to the Nigeria National Drug Policy (5.0), a target to be established is the proper disposal of expired, deteriorated and substandard drugs in 60% of public and private healthcare facilities by 2008.

The aim of the study was to develop a simple, reproducible and low-cost high performance liquid chromatography method and together with other methods, assess the quality of different brands of metronidazole tablets marketed in Abuja.

MATERIALS AND METHODS:

Drugs and Chemicals:

HPLC grade methanol (by Sigma-Aldrich Co. St. Louis USA), metronidazole reference standard powder (by Sigma Chemical Co. USA M-3761, lot 97F0147) and HPLC water. All other chemicals used were analytical grade. The drug samples were purchased randomly from five different pharmacies in Abuja.

Instrument:

Quantitative analysis was performed with high performance liquid chromatography- Agilent 1200 series coupled to a Diode Array Detector with a Reversed Phase C_{18}, 5µm, 150x46mm column and Chem Station software. The data obtained was processed using Microsoft Excel 2010. Others used in this study are a Erweka friabilator, hardness tester, Erweka disintegration apparatus, Ultratech whirl mixer, Decon F5100b sonicator, UV spectrophotometer (Schimadzu AUW220) Analytical Balance.

Aesthetic Tests:

Samples were stored as specified by the manufacturers prior use. The identification tests of the various samples were carried out by visual inspection.

Disintegration test:

The disintegration times of the various samples were determined as specified in the British Pharmacopoeia (BP) 2013 using an Erweka 6 – station disintegration tester. Distilled water was used as the disintegration medium. The results shown are the average of three determinations.

Dissolution test:

Dissolution tests were performed on the tablets using an Erweka dissolution test apparatus. The medium used was distilled water, thermostatically maintained at 37 degrees at a rotational speed of 100 rpm. Samples of 5 mL were withdrawn at 5 minutes intervals using a syringe and needle, which were replaced with fresh 5 mL of the dissolution medium after each withdrawal. The withdrawn samples were analyzed spectrophotometrically at a pre-determined wavelength of 315 nm.

Crushing force:

The crushing force of the tablets was determined with the crushing force tester (Erweka GmbH, Germany). The average hardness of 10 tablets were recorded

Friability test:

The friability of the tablets was determined with the Erweka friabilator (Erweka GmbH, Germany). Ten tablets were analyzed at 25 rpm for 4 min in all

cases, and the mean and standard deviations after triplicate determinations were calculated.

Uniformity of Weight

The uniformity of weight was carried out on 20 randomly selected tablets and average weight was determined [5].

Method Development and Validation:

During method development some important parameters were tested to ensure chromatographic separation such as the mobile phase ratio and wavelength. The optimized chromatographic condition under which separation was achieved on C18Phenyl (250 x 4.6 mm, 5µ) column using mobile phase containing mixture of methanol and water (3:17, v/v (15:85, v/v) at flow rate of 1ml/min. The wavelength was at 320 nm and the column temperature was set at 25^{o}C.

Calibration Curve:

A 10mg quantity metronidazole reference powder was accurately weighed and transferred to a 10ml volumetric flask and 5ml of 0.1MHCl was added as diluent then sonicated for 10 minutes and made up to volume with diluent to obtain a stock solution of 1mg /ml. Different concentrations in the range of 1.5 to 50µg/ml were prepared from working standard of 100µg/ml. 20µl aliquot was introduced into the column at a flow rate of 1.0ml/min, wavelength of 320nm, temperature of 25^{o}C and a mobile phase of methanol and water (Ratio 3:17, v/v) with runtime of 7 minutes in duplicate. The mean peak were plotted against respective concentrations to obtain the Calibration curve.

Samples preparation and Analysis:

Ten tablets were randomly selected, weighed accurately and the average weight determined and then triturated to a fine powder. An equivalent weight to 2.5mg of metronidazole powder was weighed and transferred to a 100ml volumetric flask, 50ml of 0.1M HCl was added sonicated for 10 minutes and made up to the mark with the diluent and then mixed. The resulting solution of strength 25µg/ml of metronidazole was filtered. 20µl aliquot was injected into the column using the same analytical conditions and parameters as used for the calibration curve in duplicate.

Method Development and Validation:

The method was developed and validated according to analytical procedure as per the ICH guidelines [6] for validation of analytical procedures in order to determine linearity, precision, LOD, LOQ and accuracy for the analyte.

Microbial Quality Assessment of Different Brands of Metronidazole Tablets:

Sample processing and analysis:

A 1g quantity of each brand of metronidazole powdered tablet was dissolved in 10 ml of sterile tryptic soy broth (TSB) and allowed to stand for 3 hrs. One milliliter of the stock was serially diluted in 9 ml of TSB up to 10^{-4}. One hundred microliter of the diluted samples were plated out on Triptic soy agar and Sabouraud Dextrose Agar (SDA) plates in duplicate and were incubated at 37°C 24 – 48 hrs for bacteria growth while the SDA plates were incubated at 25-28°C for fungal growth[7]. The colonies observed after incubation were sub-cultured on nutrient agar for purity and identification.

Identification of colonies:

The bacterial isolates were identified using standard biochemical tests [8]. The yeast cells and the fungal isolates were identified microscopically based on their cellular morphology and differentiation. The latter were stained with lactophenol - in- cotton blue dye. The total viable aerobic bacteria (TVAB) and fungal count (FC) were calculated from the number of colonies that appeared on the plates.

RESULT AND DISCUSSION:

Table 1 shows the aesthetic properties of the various brands of metronidazole tablets studied. All the brands were generally of good appeal, bitter to slightly bitter taste and white except sample T_7 which was yellow in colour. All the tablet brands had very smooth surfaces except T_2 and T_3 which also had rough edges suggestive of loss or gradual loss of integrity. **Table 2** shows the weight variation for the randomly selected 20 tablets for the respective brands. All the brands of metronidazole tablets met the BP 2013 requirement of having a RSD value of less than 5% for uniformity of weight except samples T_5 and T_7.

Preliminary experiments were carried out to achieve the best chromatographic conditions for determination of the drug substances. A C_{18} column; 150mm length and 4.6 mm inner diameter and 5µm particle size was chosen. The detection wavelength was selected as 320nm with Diode Array detector. The method was validated as per ICH guidelines by using various validation parameters such as linearity, accuracy, precision, LOD and LOQ. Chromatographic conditions were optimized by changing the mobile phase composition. Different experiments were performed to optimize the mobile phase but adequate elution of the drug could not be achieved. Eventually the best separation was obtained by the isocratic elution system using a mixture of methanol and water (15:85) at a flow rate of 1 ml/min. Under these conditions Metronidazole was eluted at 5.2 minutes with a run time of 7 minutes.

The calibration curve was linear over the concentration range of 1.5-50µg/ml with a regression coefficient of 0.999 (**Fig.2**). Reproducibility of the method was established by analysing various replicate samples of Metronidazole. The HPLC method developed was selective as it provided a good resolution with no interference from excipients. The representative chromatograms of this estimation are shown in (**Fig. 3**) and (**Fig. 4**). The HPLC method was precise and reproducible with the percentage coefficient of variation (CV %) of less than 5% as shown in **Table 4**. The Limit of Quantification (LOQ) is the lowest amount of analyte in the sample that can be quantitatively determined with suitable precision and accuracy, while the Limit of Detection (LOD) is the lowest concentration that can be detected but not necessarily quantified as an exact value. The LOQ obtained was 25ng/ml while the LOD was 15.6ng/ml as shown in **Table 5**. Hence, it may be concluded that the method is suitable for routine assay of Metronidazole raw material and formulated dosage form.

The weight uniformity test allows the formulator to check whether there is homogeneity among units from the same batch, and this is important in quality control because, it enables the formulator to avoid dose variation resulting from different weights. It has been shown severally that tablets of different weights may have different levels of active ingredients as well [9]. According to BP 2013, tablets weighing more than 250 mg should not exhibit weight variation greater than 5.0 %; consequently, results in table 2 shows that, all the tablets except T_5 and T_7 passed the weight uniformity test.

Generally all the tablets exhibit good physical properties, with none being friable. Friability test belongs to the group of tests referred to as abrasion resistance tests. Friability test specifically evaluates what happens to a tablet due to handling. If a tablet is very friable, it means that it would undergo mechanical erosion and loose integrity upon transportation. Thus, the ability of a tablet to resist friction is a very important property which ensures that the correct amount of drug is administered and the tablet integrity including appearance will not change due to handling. Generally, Tablets with losses of less than 1.0 % of the weight are considered acceptable [7]. As shown in **Table 6**, none of the eight samples showed friability higher than this limit, therefore, none failed.

The strength of a tablet during packaging, storage and transportation is usually defined by its hardness. The minimum hardness for an uncoated tablet such as those under interrogation is 4 KgF. According to **Table 6**, all the samples may be approved under this criterion, except samples T_1, T_3 and T_8. On the basis of hardness test result alone, these three brands do not have adequate mechanical strength required for normal handling, however, the remaining five samples had hardness values ranging between 6.0 and 9.0 KgF; this is a desirable attribute as tablets that are excessively hard could lead to therapeutic failure.

The disintegration process of a solid dosage form especially compressed tablets is a major step towards its bioavailability [9]. According to the pharmacopeia, an uncoated tablet must disintegrate within 15 min. On the basis of this specification, since none of the tablets disintegrated in more than 5 min, it means they all passed, implying that they passed disintegration test as stipulated in the British Pharmacopoeia for uncoated conventional tablets. **Table 6** shows the disintegration times for each

drug, showing that the results are consistent with that specified by the official compendia.

The dissolution test is generally used to predict or determine the time it would take a dosage form to release the desired amount of drug in liquids from the absorption site [9]. Before drugs can be absorbed, it must first dissolve in biological fluids; therefore, dissolution test is a very critical quality parameter for solid dosage forms. The type of excipients and/or additives as well as the formulation technology have been shown to affect the dissolution, hence absorption and consequently, the expected therapeutic effect [10, 11] of solid pharmaceutical forms for oral use. According to the official compendia, at least 85 % of the drug should be dissolved after 45 minutes of subjecting an uncoated tablet to dissolution test.

As it can be observed in **Table 6**, all the tablets dissolved and released 100 % of content in less than 45 minutes. Specifically, sample T_4 which released 100 % of the drug in ten minutes was the fastest, while T_3 had the longest time of 34 minutes. It is important to note that, the dissolution result of the tablets is reflective of the disintegration profile of tablets. Worthy of note too is the unusually high amount of drug contained and released from T_5 and T_7. Although the observation was not unexpected because of the corresponding high weight variation, but, the clinical implication of this, is drug overdose and exaggerated adverse effects.

The results of the microbiological quality study are represented on **Table 8**. From the results the total viable aerobic bacterial count ranged from 1.0 x 10^2 – 1.5 x 10^2cfu/g. The tablets were free from fungal contamination. The pathogenic organisms isolated include *Bacillus sp.*

The microbial quality of pharmaceutical products may be influenced by the environment in which they are manufactured, the raw materials used in their production with the exception of preparations which are terminally sterilized in their final containers. The presence of *Bacillus sp* in the brand assessed was not unexpected as aerial contamination of hospital pack products during dispensing, transportation and storage is a common phenomenon. However, the bioburden level fell within the acceptable limit for *Bacillus sp.* and *S. aureus* for oral (non-aqueous) dosage form as specified in the United States Pharmacopoeia (USP). According to USP 2013, *Escherichia coli* must not be found in pharmaceutical products, whereas, total viable bacterial and yeast counts must not exceed 10^2cfu/mL[12].

TABLE 1: AESTHETIC *TEST*FOR DIFFERENT BRANDS OF METRONIDAZOLE TABLETS

Sample Code	Colour	Shape	Lustre	Nature of Surface
T_1	White	Circular convex surface tablets	Shiny	Smooth
T_2	White	Circular convex surface tablets	Dull	Smooth with rough edges
T_3	White	Circular convex surface tablets	Shiny	Smooth with rough edges
T_4	White	Circular convex surface tablets	Shiny	Smooth
T_5	White	Circular convex surface tablets	Shiny	Smooth
T_6	White	Circular convex surface tablets	Shiny	Smooth
T_7	Yellow	Circular convex surface tablets	Shiny	Smooth
T_8	White	Circular convex surface tablets	Shiny	Smooth

TABLE 2: UNIFORMITY OF WEIGHT

Sample Code	Average weight (mg)	Variance	Standard Deviation	Relative Standard Deviation (%)
T_1	342.45	$1.302x10^{-5}$	0.0036087	1.05
T_2	375.62	$1.165x10^{-4}$	0.0107959	2.87
T_3	350.99	$1.301x10^{-4}$	0.0114092	3.25
T_4	504.15	$1.054x10^{-4}$	0.0102689	2.03
T_5	513.18	$8.770x\ 10^{-3}$	0.0312250	6.08
T_6	507.14	$2.090x\ x10^{-5}$	0.0045780	0.90
T_7	536.49	$1.178x\ x10^{-3}$	0.0343492	6.40
T_8	373.62	$7.920x10^{-5}$	0.0028157	0.75

TABLE 3: OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS AND SYSTEM SUITABILITY PARAMETERS.

Parameter	Chromatographic conditions
Instrument	Agilent Technologies 1200 series with ChemStation software
Column	C18 150mm x 4.6 mm ID x 5µm
Detector	Diode Array Detector
Diluents	0.1M HCl
Mobile Phase	Methanol: Water (15:85)
Flow rate	1.0ml/min
Detection wavelength	320nm 16BW
Temperature	25^{o}C
Injection volume	20µl
Retention time	5.2 min.

TABLE 4: INTRADAY AND INTER-DAY PRECISION FOR METRONIDAZOLE DETERMINATION

Parameter	Concentration (µg/ml)	%Coefficient Variation
Intraday run	6.250	2.21918
	25.000	4.48973
Inter day run	6.250	2.8068
	25.000	0.9031

TABLE 5: LIMIT OF DETECTION AND LIMIT OF QUANTITATION

	Retention Time (min)	Peak Area (mm^2)	Concentration Value (ng/ml)
LOD	5.201	-	15.625
LOQ	5.207	13.38967	25.000

TABLE 6: PHYSICAL AND RELEASE PROPERTIES OF METRONIDAZOLE TABLETS.

Sample	Disintegration time (min)	Dissolution (%)	Friability (%)	Hardness (KgF)
T_1	0.58 ± 0.14	100.71 (T_{12})	0.24 ± 0.01	3.00 ± 0.00
T_2	0.73 ± 0.07	110.04 (T_{23})	0.94 ± 0.10	6.40 ± 0.90
T_3	1.37 ± 0.63	100.21 (T_{34})	0.42 ± 0.04	3.00 ± 1.00
T_4	2.02 ± 0.55	100.82 (T_{10})	0.51 ± 0.08	9.80 ± 0.84
T_5	3.21 ± 1.40	122.08 (T_{11})	0.42 ± 0.08	8.60 ± 0.55
T_6	4.66 ± 0.25	110.39 (T_{28})	0.32 ± 0.00	6.00 ± 0.71
T_7	1.55 ± 0.37	131.82 (T_{12})	0.99 ± 0.00	9.00 ± 1.41
T_8	0.75 ± 0.03	100.34 (T_{27})	0.75 ± 0.00	0.00 ± 0.00

TABLE 7: PERCENTAGE CONTENT OF METRONIDAZOLE IN DIFFERENT BRANDS METRONIDAZOLE TABLETS

Sample	Amount of Drug Declared (mg)	Average Amount of Drug determined (mg)	Percentage Content (%)	Remark
T_1	200	175.0154	87.50769	Pass
T_2	200	183.1046	91.55228	Pass
T_3	200	220.8197	110.4099	Pass
T_4	200	214.7846	107.3923	Pass
T_5	200	211.1904	105.5952	Pass
T_6	200	211.5817	105.7909	Pass
T_7	200	213.2835	106.6418	Pass
T_8	200	202.1677	101.0838	Pass`

TABLE 8: BIOBURDEN OF METRONIDAZOLE TABLETS

Sample	TVAB (cfu/g)	FC (cfu/g)	Micro-organisms
T1	-	-	-
T2	-	-	-
T3	-	-	-
T4	1.5 x 10^2	-	*Bacillus sp.*
T5	-	-	-
T6	-	-	-
T7	-	-	-
T8	-	-	-

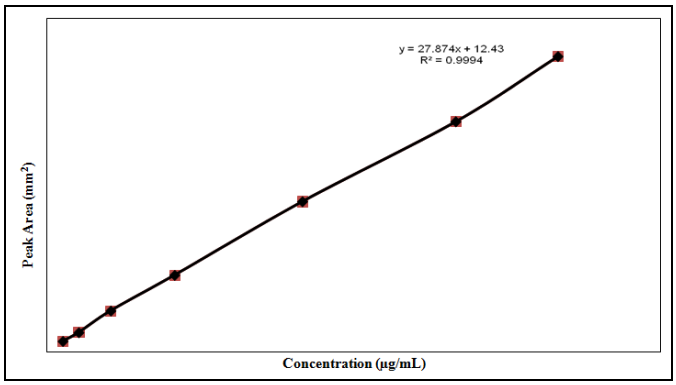

FIG. 2: CALIBRATION CURVE OF METRONIDAZOLE

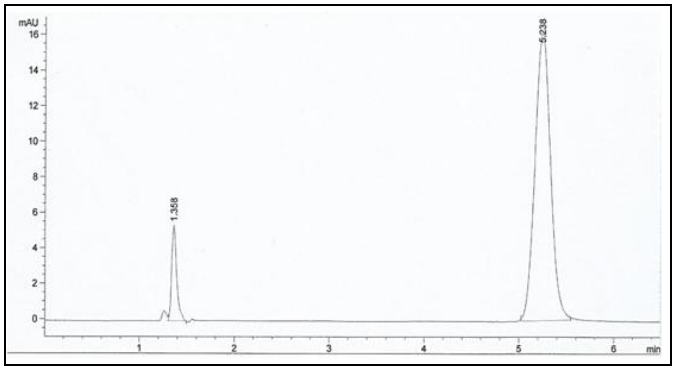

FIG. 3: CHROMATOGRAM OF METRONIDAZOLE REFERENCE

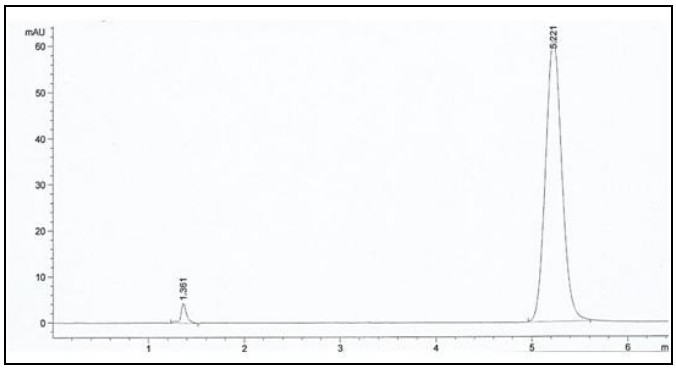

FIG. 4: CHROMATOGRAM REPRESENTATIVE METRONIDAZOLE

CONCLUSION: A simple, accurate and cost effective Reverse Phase HPLC method was developed for the quantitative determination of metronidazole in tablets. The LOD and LOQ were found to be 15.6ng/ml and 25ng/ml respectively.

The coefficient of variation for intra and inter-day runs were less than 5%. The calibration curve was linear with a regression of r = 0.9994 as shown in **Fig.2**. The HPLC method developed was selective as it provided a good resolution with no interference by the excipients and the retention time of metronidazole in this method was 5.2 minutes. All the eight different brands of metronidazole tablets fell within the acceptable limit range of 85-115% [5] as shown in Table 7. From the result obtained from the microbial load determination, there is a need for constant monitoring of marketed drugs within the country to ensure that commercially available drugs in markets conform with the Pharmacopeia standards, so as to meet up with national health delivery policy in Nigeria. The developed HPLC method can be employed for the routine quality control analysis of metronidazole.

CONFLICT OF INTEREST: There is no conflict of interest.

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