Biowaiver studies of Metronidazole tablets (400mg): An alternative to *In-vivo* bioequivalence Studies

Kashif Ali Safdar[1], Syed Baqir Naqvi[1], Shahnaz Usman[2*], Saeed-ur-Rehman[3] and Iyad Naeem Muhammad[1]

[1]Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi, Pakistan
[2]RAK College of Pharmaceutical Sciences, RAK Medical & Health Sciences University, Ras Al Khaimah, UAE
[3]Getz Pharma Private Limited, Karachi, Pakistan

Abstract: The aim of the study was to investigate the dissolution behavior of commercially available brands of metronidazole and to provide basic tool to evaluate the comparative effectiveness and interchangeability of generic brands under biowaiver conditions. The dissolution test for six brands of metronidazole 400mg tablets was performed and physical controls were analyzed. Basket Rack methods at 100rpm were used to estimate release pattern of drug. Pharmaceutical parameters of tablets were analyzed. In order to evaluate dissolution profiles, multiple point dissolution were performed and calculated 85.96±0.41 to 90.56±0.93 % within 15 minutes in pH 1.2,85.50±1.40 to 88.99±0.80% in pH 4.5 and 85.37±1.94 to 92.79±0.89% in pH 6.8 dissolution medium respectively. Five different kinetics have been studied to predict and evaluate the acceptability level of drug release. The results show that Hixson-Crowell, first-order and Weibull demonstrated the drug release with $R^2 \geq 0.95$ that predicted the tablets were pharmaceutically equivalent. One-way ANOVA at $p \geq 0.05$ level and similarity factors (f2) were used to estimate the discrepancy and intimacy among the brands. It is a need of time to constantly monitor the marketed generic drugs products and their release profiles to confirm their in vitro bioequivalence which can help to reduce the time, cost and unnecessary exposure of healthy subjects to medicines.

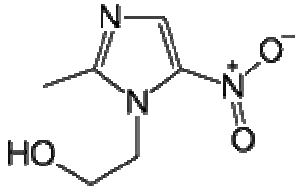
Keywords: Metronidazole, dissolution behavior, comparative study, biowaiver studies.

INTRODUCTION

Metronidazole is an oral synthetic antiprotozoal and antibacterial agent. It is commonly used in the treatment of acute intestinal amebiasis (amebic dysentery) and amebic liver abscess caused by *Entamoeba histolytica* or *Giardia lamblia* (Sweetman 2009; Metronidazole, 2015; Amebiasis, 2015). It has excellent tissue penetration and bactericidal activity makes it useful for deep-seated infections. But WHO International Agency for Research on Cancer *(IARC, 2010),* indicated that metronidazole also comes under the category of carcinogenic drug. A study also demonstrated the development of chromosomal abnormalities in circulating lymphocytes if treated with metronidazole in patient with Crohn's disease (MMPI, 2013).As per FDA, the Waiver request of in vivo testing for capsule (oral) is "not applicable" whereas for tablet (oral) is acceptable on the basis of bioequivalence studies on the 500 mg strength or on the basis of *in vitro* dissolution testing of all strengths.

It is listed that almost 13 pharmaceutical companies in Karachi (Pakistan) are engaged in manufacturing of 32 brands of generics metronidazole oral. It is also reported that various substandard medicines are freely available in local markets. Under such critical prominences of metronidazole the availability of these brands make it difficult to select good one for the patient. The comparative dissolution studies help to determine similarity of generic to the innovator medicine. When manufacturing a generic medicine, demonstration of dissolution profile is needed to evaluate and interpret their comparative data. In practice, the r*outine in vivo* measurement of the drug in blood and urine is not possible. Comparative dissolution testing of generic drug product provides a considerable economic support in terms of cost and time to launch a new drug product in the market (Shahnaz, *et al.*, 2014).

As per Biopharmaceutical classification system (BCS), Class 1 drugs are considered as highly soluble and highly permeable. Metronidazole falls in Class I BCS (Camila *et al.,* 2011). It is expected that it will not cause any bioavailability problems. Under such situation a bioequivalence study may be waived based on case history and similarity of dissolution profiles. Chemical structure of drug (BP, 2009).



The aim of the present work, as a surveillance study, was to assess the quality of different brands of metronidazole

**Corresponding author:* e-mail: shahnazgauhar@gmail.com

tablets (400mg) available in the local market to determine the appropriateness of their inter-changeability. Also to investigate the effect of dissolution medium such as pH 1.2, 4.5 and 6.8 buffers, on the release of drug from immediate release tablet by establishing the bioequivalence and to provide basic tool for biowaiver filing.

MATERIALS AND METHODS

Materials

Metronidazole was obtained as gift sample from Sanofi-Aventis Pakistan limited. Potassium Chloride (Fischer Chemical), hydrochloric acid (Merck), Sodium Acetate Trihydrate (Sigma Aldrich), Acetic Acid (Glacial, Merck), Potassium Dihydrogen Phosphate (Merck), Sodium Hydroxide (Sigma Aldrich) and distill water was freshly prepared by distillation method. All six brands of metronidazole tablets were collected from retail market of Karachi (Pakistan) which was within product expiration dates (table 1).

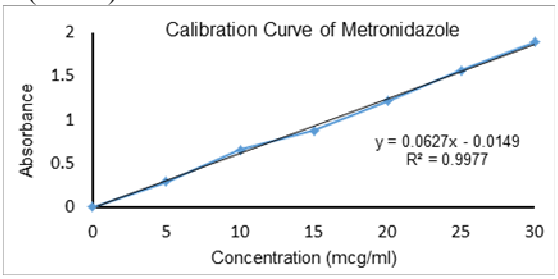


Fig. 1: Calibration Curve (Linearity) of Metronidazole

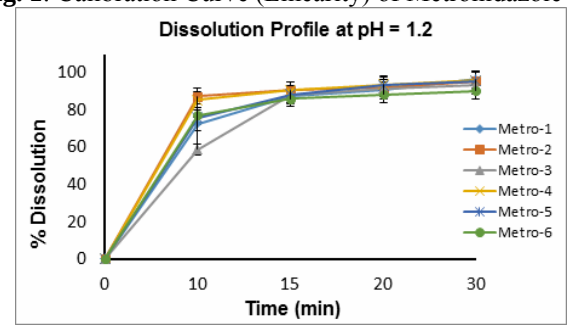


Fig. 2: Graphical Presentation of % Dissolution Data of Metronidazole Tablets in pH 1.2 buffer

Instruments

The entire analysis was carried by using dissolution apparatus (Agilent Technologies; Model: DS-708; With Auto-sampler, USA), UV/Visible Spectrophoto- meter (Shimadzu; Model: UV-1800, Japan), Analytical Balance (Mettler Toledo; Model: XS-105, Switzerland), Fume Hood (LabTech, Korea), and pH Meter (Mettler Toledo; Model: S220, Switzerland).

Method

Preparation of Dissolution Mediums (DM): All three test medium of pH 1.2, 4.5 and 6.8 were prepared as specified in the USP / BP.

pH 1.2 Buffer: Weighed accurately 18.64g of potassium chloride and was dissolved in distilled water. The pH was adjusted with HCl to 1.2 and finally volume was adjusted with distilled water up to 5 Liter.

pH 4.5 Buffer: Weighed accurately 14.95g of sodium acetate trihydrate and was dissolved in distilled water. The pH of4.5 was adjusted with acetic acid and finally volume was adjusted with distilled water up to 5Liter.

pH 6.8 Buffer: Weighed accurately 34.025g of potassium dihydrogen phosphate and was dissolved in distilled water. The pH was adjusted to 6.8 by sodium hydroxide solution prepared by dissolving 5g in 200mL distill water and finally volume was adjusted with distilled water up to 5 Liters (USP 35, 2012).

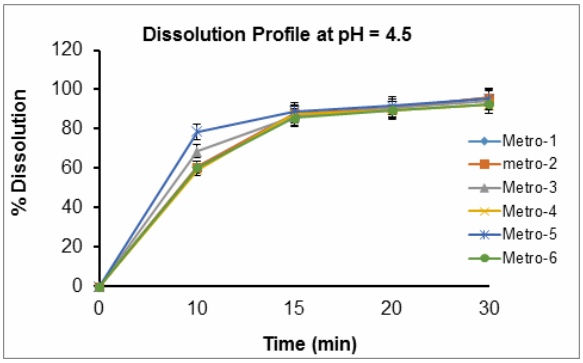
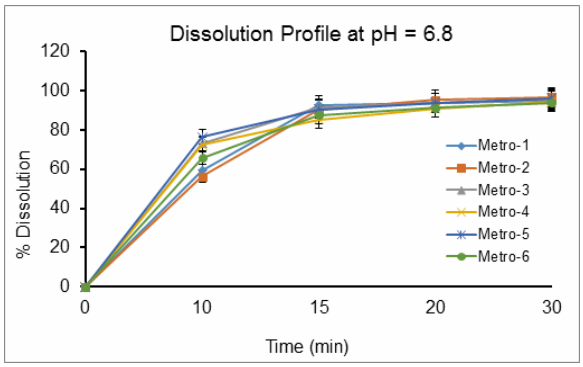


Fig. 3: Graphical Presentation of % Dissolution Data of Tablets in pH 4.5 buffer



Result based on n = 6

Fig. 4: Graphical Presentation of % Dissolution Data of Tablets in pH 6.8 buffer

Calibration curve of metronidazole

The calibration curves were constructed for three dissolution medium with different concentrations (5-30 µg/ml) by using pure metronidazole powder. Serial dilution of stock solutions was made for pH 1.2, 4.5, and 6.8 respectively. The absorbance of solutions were measured using UV spectrophotometer (fig. 1).

Standard preparation

Metronidazole (44.4mg) powder was weighed accurately and transferred carefully in to 100mL volumetric flask. It

was diluted with1.2, 4.5 & 6.8 buffers separately up to the volume of 100mL. After proper shaking, 5ml of solution was pipetted out and was diluted to 100ml to get the final concentration of 22.2µg/ml. Filter through 0.45µm (pore size) filter paper.

Sample preparation

Six tablets of each brand were dissolved in 900ml of respective dissolution medium (pH 1.2, 4.5 and 6.8 buffers) separately. Five (5) milliliter of solution was diluted with DM to get the final concentration of 22.2µg/ml and spectrophotometrically measured at λ = 278nm.The concentration in each sample was calculated from standard calibration curve. The curve of metronidazole was prepared in 0.1N HCl, 4.5 acetate buffers, and 6.8 phosphate buffer separately.

Dissolution testing procedure

The apparatus USP Type-I was adjusted at temperature 37±0.5ºC and 900ml of DM was transferred separately each time to the dissolution vessel. Apparatus was run at 100 rpm and 10ml sample was withdrawn at time intervals of 10, 15, 20 and 30 minutes without replacement. Samples collected were filtered through 0.45µm filter paper, and diluted up to 100ml. Average and cumulative drug release percentages were calculated for dissolution profile estimation.

$$\% \text{ Dissolution} = \frac{\text{Absorbance of Sample}}{\text{Purity of Standard}} \times \times \frac{\text{Conc. of Standard}}{\text{Conc. of Sample}} \times \frac{\text{Purity of Standard}}{100} \times 100$$

STATISTICAL ANALYSIS

Analysis of Variance (ANOVA) One-way and f2 similarity factor were used to compare the dissolution profiles of six tablets for each batch. In all cases, a value of $p<0.05$ was considered significant. The dissolution profile were also applied to different kinetic models as presented in table 2

RESULT

Pharmaceutical evaluation of tablets

The evaluation of quality attributes was done on different brands of metronidazole tablets purchased from the local market by running the series of tests defined by the pharmacopeias. The summary of quality control parameters of tablets are presented in table 3.

Comparative dissolution profiling (CDP)

The CDP of metronidazole tablets were performed in three dissolution mediums separately. The samples were withdrawn at different time intervals and the absorbances were noted. Dissolution profile was compared and evaluated with the help of Microsoft Excel add-in "DD Solver" v1.0. Fig. 2 shows % dissolution release of 6 brands in dissolution medium of pH 1.2, fig. 3 for pH 4.5 and fig. 4 for pH 6.8 buffers.

STATISTICAL ANALYSIS

The dissolution profile was evaluated with ANOVA to calculate the general differences among means of batches (table 7). Results were conformed to F value by calculating the variation between two values as equal and unequal. It helps to determine which specific groups differed from each other (table 8). Whereas Microsoft Excel add-in "DD Solver" v1.0 was used to calculate similarity factor f_2 for different brands of tablets in different pH (table 9).

DISCUSSION

Instead of conducting an expensive and time consuming *in vivo* studies during drug development a dissolution test could be adopted as the surrogate basis to determine as to whether the two pharmaceutical products are equivalent or not (CDER, 1995; FDA, 1997 and EMEA, 2009). The risk of therapeutic inequivalence of two immediate release products can never be reduced to zero, even if a full clinical study is performed.

Metronidazole is concentration-dependent antibiotic, its efficacy against the elimination of H. pylori within the intestinal lumen, or effective killing of anaerobes at any systemic sites of infection, both are dependent on the rapid release of the drug from its formulation (tablets), and also on the dissolution of the drug in the gastrointestinal fluids to obtain therapeutic concentration of the drugs, which could then perform locally or systematically (Sefunmi, 2014).

The pharmaceutical analysis of the studied brands of metronidazole was done to estimate the quality control parameters that help to predict the pharmaceutical equivalency of the formulations. All the brands were under their expiry date. All formulations evaluated had suitable organoleptic properties. The weight variations of tablets were determined to assess indirectly the content uniformity of the drug within the batches. Hardness tester was used to estimate the hardness of ten tablets of each brand. No major divergence was observed in the hardness of tablets. Five selected brands were film coated only one brand (Metro-6) was uncoated and friability test was carried out on it and was found 0.73% (table 3).

The disintegration test was done on study brands as per the USP. Disintegration time of all tablets was found well within the acceptance criteria (NMT 15 minutes for uncoated tablet & NMT 30 minutes for film coated tablet). The disintegration time of 400mg tablets were within 6.5±0.31-14.4±0.23 minutes with no significant differences among the six tablets of each brand (table 3). A newly developed and validated HPLC analytical method was used to analyze the drug content (Kashif, *et al.,* 2014). It was found to be 96.98±0.23-100.53±0.55,

well within the acceptance criteria defined by the USP (NLT 90% and NMT 110%). HPLC System suitability analysis was conducted before starting the assay testing.

The focus of this study was to determine the effect of different dissolution medium on the release of metronidazole in immediate release formulations. As per CDER, 1997 dissolution testing is required for all solid oral dosage forms and it is useful for the development of products and stability study. According to the BCS guidelines, *in vitro* dissolution testing is a useful tool to forecast the *in vivo* performance of drug products and reduce the required number of bioavailability/ bioequivalence studies.

The dissolution test was performed on all metronidazole tablets separately. Percentage dissolution was calculated to assess the release of drug as per the acceptance criteria set by the FDA and WHO for biowaiver studies. The FDA guidance on dissolution testing for immediate release solid oral dosage forms includes the use of the BCS guidelines for biorelevant dissolution tests, which is based upon API solubility and permeability (Wang, *et al.,* 2009)

Three different dissolution mediums such as pH 1.2, 4.5 and 6.8 buffers, were used for the dissolution profiling of metronidazole (400mg) tablets in 900 ml at 37±0.5°C. The samples were taken at different time of interval i.e. at 10, 15, 20 and 30 minutes and absorbance was obtained at λ_{max} 278.0 nm. Microsoft Excel add-in "DD Solver" v1.0 was used to calculate the percentage dissolution of the drug. The percent release of metronidazole was between 85.96±0.41 to 90.56±0.93, 85.50±1.40 to 88.99±0.80 and 85.37±1.94 to 92.79±0.89 within 15 minutes in 1.2, 4.5 and 6.8 pH DM respectively (fig. 2, 3 & 4). The results were supported with the work of Sefunmi (2014), Nallagundla *et al.,* (2014) and Kahaliw & Ashenef, (2013).

Numbers of models have been proposed by different scientists to calculate the release pattern and kinetics of drugs. Different models used for the data evaluation were zero order, first order, Higuchi, Hixson Crowell and Weibull. In the present study, DD Solver v1.0 was used to calculate and analyze the drug profile. All the six brands of 400 mg tablets failed to obey the zero order kinetics and higuchi with R^2 is ≤0.95 (not acceptable) in pH 1.2, 4.5 and 6.8 buffers respectively. Whereas the drug release were adequately described by Hixson-Crowell, first-order and Weibull with R^2 value ≥0.95 in pH 1.2, 4.5 and 6.8 buffers separately that is used to define pharmaceutical equivalency of different generic brands.

Tables 4, 5 and 6 shows that the R^2 for Weibull model is similar in all three media and is almost equal to 1 indicating that the shape of the curve corresponds exactly to the shape of an exponential profile. Weibull curves define the actual distribution of dissolution data. The value for β was <1 i.e. 0.147-0.385 in pH=1.2, 0.153-0.328 in pH =4.5 and 0.094-0.412 in pH=6.8 that indicated the failure rate decreased with time. This helps to estimate the amount of drug dissolved as a function of time.

Statistical analysis was done by using one-way ANOVA to compare the drugs profile of six brands of metronidazole tablets to estimate the variation among the brands and to determine whether any of these means are statistically significantly different from each other. In tables 7, the mean sum of squares (SS) is indicating the variance between the brands and mean sum of squares (MSE) is estimating the variance within the brands. The table value of F at 0.05 levels of significance for 5 and 24 is 2.621 for all three DM whereas the calculated value of F is smaller than that which indicates that there is no significant difference in samples means (table 8).

As per the FDA guideline the similarity factor, *f2* is not needed when greater than 85% of the labeled amount of drug is dissolved within 15 min when tested in 0.1 N HCl, 4.5 acetate buffers, and 6.8 phosphate buffer. In the present study to verify the similarity factors metro-1(400 mg) tablets were taken as reference drug and similarity factor f_2 was calculated with the help of "DD Solver" v1.0. F_2 values of all brands were found to be within the acceptance limit in all three medium i.e. greater than 50. The range of similarity in pH 1.2 medium were 58.21-82.96, in pH 4.5 it was 54.30 to 83.58 and in pH 6.8 was 55.35-83.02 with % CV of 6.21-18.11 (table 9). The results show that substitution of generic can be made for innovator in clinical use.

CONCLUSION

In conclusion it was revealed that all the six commercial brands (national and multinational) of metronidazole film coated/ uncoated tablets in Karachi markets, met the official specification of USP pharmacopeia. The results of the study show the similarity in release profile of metronidazole from the branded and generic product that can helps to estimate the effectiveness and clinical outcome of medicines. The attempt was made to conduct an *in-vitro* dissolution study of drugs with different pH buffers that cover the *in-vivo* environments of GIT as recommended by FDA for biowaiver study. Different independent and dependent models were applied to analyze dissolution profiles.

Different metronidazole dosage forms are included in the essential drug list (EDL, 2013), so it is vital that the products should be rigorously tested to avoid possible serious consequences for patients. If such products are found to be substandard, the testing would be able to keep the check and balance on quality of generic drug products.

Table 1: Demographic detail of different brands of Metronidazole (400mg) Tablet

S. No.	Brand Name	Batch No.	Tablet Color	Tablet Shape	Coating Description
1.	Metro-1	WA248	Yellowish	Round	Film Coated
2.	Metro-2	8W69	Orange like	Round	Film Coated
3.	Metro-3	142	Bluish	Oblong	Film Coated
4.	Metro-4	1815	Yellowish	Oblong	Film Coated
5.	Metro-5	2401	Yellowish	Round	Film Coated
6.	Metro-6	36	Yellowish	Round	Uncoated

Table 2: Dissolution Models with their Equations

Model Name	Equation
Zero Order	$F=k_0xt$
First Order	$F=100x[1-Exp(-k1xt)]$
Higuchi	$F=kHxt^{0.5}$
Hixson Crowell	$F=100x[1-(1-kHCxt)^3]$
Weibull	$F=100x\{1-Exp[-((t-Ti)^{\beta})/\alpha]\}$

Table 3: Pharmaceutical parameters estimation of Metronidazole Tablet

Batch No.	Weight Variation (Mean ± SD)	Thickness (mm)	Hardness (Kp)	Friability % w/w	Disintegration Time (Sec)	Drug Content (%)
Metro-1	520.1± 6.27	5.26±0.05	12.7±1.3	Film Coated	10.3 ±0.21	100.53 ±0.55
Metro-2	594.2±8.51	5.07±0.05	12.8±1.6	Film Coated	13.4±0.16	97.91 ±0.21
Metro-3	758.8±12.34	5.84±0.07	13.7±3.5	Film Coated	14.4±0.23	96.98 ±0.23
Metro-4	695.8±16.91	5.12±0.06	9.5±1.9	Film Coated	6.5±0.31	100.01 ±0.34
Metro-5	524.9±7.52	5.39±0.06	11.2±1.7	Film Coated	12.6±0.26	98.91 ±0.47
Metro-6	571.9±11.94	5.09±0.12	8.1±1.4	0.73%	11.6±0.33	98.77±0.03

Table 4: Dissolution Data evaluated with kinetic Models in pH 1.2 buffers

Parameter	Metro-1	Metro-2	Metro-3	Metro-4	Metro-5	Metro-6
Zero Order - Model						
k_0	4.148	4.270	4.023	4.282	4.191	4.022
R^2	0.6106	0.4319	0.6807	0.4605	0.5633	0.4921
First Order - Model						
k1	0.130	0.181	0.109	0.175	0.141	0.132
R^2	0.9982	0.9898	0.9789	0.9942	0.9973	0.9829
Higuchi Model						
kH	20.017	20.825	19.300	20.852	20.290	19.554
R^2	0.9419	0.8642	0.9411	0.8791	0.9231	0.8939
Hixson-Crowell Model						
kHC	0.033	0.042	0.029	0.041	0.035	0.034
R^2	0.9915	0.9754	0.9821	0.9830	0.9912	0.9645
Weibull Model						
A	1.059	1.029	0.619	1.044	1.118	0.681
B	0.385	0.330	0.173	0.348	0.357	0.147
Ti	7.713	0.391	9.952	3.511	7.004	8.770
R^2	0.9995	0.9998	0.9998	0.9998	0.9998	1.0000

Table 5: Dissolution Data evaluated with kinetic Models in pH 4.5 buffers

Parameter	Metro-1	Metro-2	Metro-3	Metro-4	Metro-5	Metro-6
Zero Order Model						
k_0	4.069	4.028	4.079	4.191	3.971	3.974
R^2	0.7006	0.5981	0.6317	0.5297	0.6779	0.6732
First Order Model						
k1	0.112	0.120	0.120	0.147	0.106	0.107
R^2	0.9827	0.9891	0.9955	0.9958	0.9837	0.9795
Higuchi Model						
kH	19.492	19.449	19.656	20.331	19.056	19.077
R^2	0.9511	0.9293	0.9452	0.9105	0.9458	0.9402
Hixson-Crowell Model						
kHC	0.029	0.031	0.031	0.037	0.028	0.028
R^2	0.9865	0.9806	0.9904	0.9862	0.9817	0.9787
Weibull Model						
A	0.818	0.717	0.971	0.926	0.750	0.623
B	0.297	0.180	0.328	0.303	0.223	0.153
Ti	9.606	9.409	8.454	7.397	9.770	9.968
R^2	0.9996	0.9996	0.9997	0.9997	1.0000	0.9999

Table 6: Dissolution Data evaluated with kinetic Models in pH 6.8 buffers

Parameter	Metro-1	Metro-2	Metro-3	Metro-4	Metro-5	Metro-6
Zero Order - Model						
k_0	4.133	4.210	4.143	4.238	4.073	4.093
R^2	0.6547	0.5725	0.7152	0.5557	0.6396	0.5971
First Order - Model						
k1	0.120	0.140	0.113	0.147	0.119	0.127
R^2	0.9611	0.9951	0.9640	0.9972	0.9888	0.9959
Higuchi Model						
kH	19.837	20.365	19.806	20.524	19.609	19.767
R^2	0.9167	0.9230	0.9386	0.9195	0.9396	0.9355
Hixson-Crowell Model						
kHC	0.031	0.035	0.030	0.037	0.031	0.033
R^2	0.9699	0.9934	0.9774	0.9931	0.9867	0.9873
Weibull Model						
A	0.443	0.552	0.747	0.722	0.776	1.457
B	0.094	0.177	0.319	0.282	0.250	0.412
Ti	9.999	9.753	9.720	8.682	9.044	6.459
R^2	1.0000	0.9999	0.9997	0.9999	0.9999	0.9997

Table 7: Analysis of Variance For *in vitro* dissolution of tablets in pH 1.2, 4.5 and 6.8DM

DM	Source of Variation	SS	df	MS	F	P-value	F crit
pH = 1.2	Between Groups	177.5823	5	35.5165	0.02217	0.99976	2.621
	Within Groups	38448.45	24	1602.02			
pH = 4.5	Between Groups	96.06883	5	19.2138	0.012426	0.99994	2.621
	Within Groups	37110.49	24	1546.27			
pH = 6.8	Between Groups	63.12799	5	12.6256	0.007742	0.99998	2.621
	Within Groups	39139.49	24	1630.81			

At the 0.05 level, NOT significantly different

Table 8: Calculated value F

F	Metro-1	Metro-2	Metro-3	Metro-4	Metro-5	Metro-6
	Ref	1.007	0.942	1.065	0.939	0.980
T-Stat Two-Sample Assuming Equal Variances	Ref	-0.046	-0.144	0.043	-0.143	-461
T-Stat Two-Sample Assuming Unequal Variances	Ref	0.121	-0.144	0.043	-0.143	-0.046

Table 9: Similarity f_2 Data of Metronidazole Tablet Brands

Dissolution Medium	F_2 Values				
	Metro-2	Metro-3	Metro-5	Metro-4	Metro-6
pH 1.2	58.21	59.71	82.96	60.87	71.36
pH 4.5	69.09	70.40	83.49	54.30	83.58
pH 6.8	59.97	83.02	69.47	55.35	57.89
Mean	62.42	71.04	78.64	56.84	70.94
% CV	9.36	16.43	10.11	6.21	18.11

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