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AN *IN-VITRO* NEW BIOEQUIVALENCE STUDY AND DENSITOMETRIC METHOD FOR DETERMINATION OF AZITHROMYCIN TABLETS OF DIFFERENT BRANDS

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ABSTRACT

Bioequivalence study of azithromycin of three brands (local, regional and national) was investigated by this new *In-vitro* densitometric method. The proposed method depends on measuring the optical density of the inhibited zone area of *Klebsiella pneumonia* and *Staphylococcus aureus* caused by the incubation of azithromycin using highly resolution digital camera and Image J software. Good correlations were obtained between the inhibited zone area and the antibiotics concentrations (0.993-0.994). The limits of detection and limits of quantitation were 2.23, 6.69 and 2.22,6.66µg/mL with *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively. The proposed method was validated according to US-Food and drug administration (FDA) guidance for bioanalytical method validation and USP 31 guidelines. The bioequivalence study revealed that all the investigated dosage forms brands are bioequivalent.

KEYWORDS

Azithromycin, Densitometric method, Antibacterial test, *In-vitro* bioequivalence, Tablets.

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INTRODUCTION

Azithromycin chemically named 2R,3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R) -2- Ethyl -3, 4, 10 trihydroxy-3, 5, 6, 8, 10, 12, 14-heptamethyl - 15 - oxo - 11 - {[3, 4, 6 - trideoxy-3 (dimethylamino)-β-D-xylo-hexopyranosyl]oxy}-1-oxa-6 azacyclopentade can-13-yl 2, 6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranoside is a macrolide antibiotic, it's mechanism of action

throughout binding irreversibly to a site on the 50S subunit of the bacterial ribosome, thus inhibiting the translocation steps of protein synthesis. Azithromycin has a strong activity against respiratory infections due to *H. influenzae* and *Moraxella catarrhalis*. Azithromycin is now the preferred therapy for urethritis caused by Chlamydia trachomatis[1], it's chemical structure as in Figure No.1. This drug is used also for the treatment of a number of bacterial infections including middle ear infections, strep throat, pneumonia, traveler's diarrhea and certain other intestinal infections[2]. A simulation study revealed that this drug can be used for treatment of COVID-19[3]. Some analytical methods have been reported for bioequivalence study of azithromycin capsules or tablets includes: RP-HPLC method[4], LC-MS/MS[5,6]. It is clear that the used methods of analysis are time consuming and expensive, in addition involving volunteers, so this proposed *in-vitro* cheap accurate densitometric route was developed which depending on image capturing and treated with Image J software.

EXPERIMENTAL

MATERIAL AND REAGENTS

Twenty grams of Muller Hinton agar (Tulip Diagnostic, India) was weighed and dissolved in 500mL of distilled water and spread equally over the surface of 20 petri-dishes using a spreader. 6mm - diameter discs (made in England) were made and placed on the cultured media surface. Azithromycin (purity is 100.45%) was obtained from Shafaco Pharmaceutical Ind. (Sana'a- Yemen) as a gift. Azicure®, azithromycin IBN Hayyan®, maxazi® tablets; each brand contains 500mg/tablet were bought from the market.

Preparation of standard solution

Stock solution containing 100µg/mL of azithromycin was prepared in ethanol and successive dilutions were prepared to form 5, 10, 20 and 40µg/mL of the drug into 10mL conical flasks.

Preparation of samples solution (tablets)

Ten tablets were taken, grounded and average weight of a tablet was calculated, the powder equivalent to 10mg was taken in a 100mL volumetric flask. Suitable dilutions were taken to prepare solutions containing 10 and 20µg/mL into 10mL conical flasks. The contents of the flasks were sonicated for 15 minutes and the volume completed to the mark with ethanol.

General procedure of the antibacterial activity measurement

The antibacterial activity test was carried out using Kirby-Bauer antibiotic testing (KB testing or disc diffusion antibiotic sensitivity testing)[7]. After preparation of the Mueller Hinton agar by dissolving suitable amount in distilled water at boiling temperature for 5-15 minutes. A quantity of the medium poured into the petri-dishes to form a uniform layer 4mm in thickness. Store the dishes to ensure that the surface of the medium is dry at time of use. Swapping a suitable amount of *Staphylococcus auras* and *Klebsiella pneumonia* into separated petri dishes by inoculating loop. 10µL volume of standard and samples working solution added to each filter paper disc. Impregnate the disc paper with the solutions of the reference substance or solutions of the sample to be examined and place on the surface of the agar. Incubate at 37±2°C for night (nearly about 24 hr). The image of inhibition zone was taken using Galaxy S6 (the resolution 16MB) from a distance about 30cm. Then the images (in the tiff form) treated with Image J software. The parameter that used was inhibited zone area which calculated by Image J software. Four concentrations of standard azithromycin (5, 10, 20 and 40µg/mL) per petri dish and two concentrations of the three brands tablets containing azithromycin (10 and 20µg/mL of each brand), see Figure No.2.

RESULTS AND DISCUSSION

Method validation

The proposed densitometric method was validated in according to US-Food and drug administration (FDA)[8] and USP 31 guidelines[9]. Analysis of statistic data was done using Excel 2003 (Microsoft Office). The studied validation parameters were;

Calibration and linearity

The linearity of the method was in the range 5-40µg/mL for azithromycin against both types of

the investigated bacteria. Good correlations were obtained between drug concentration and the inhibition zone area, the correlation coefficients were 0.994 and 0.993 for azithromycin against *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively.

Detection limit LOD and quantitation limit LOQ

The minimum inhibitory concentration (MIC) was determined for the studied drug. MIC was 5µg/mL. According to mathematic calculations, when the concentration of the antibiotic closure to the zero the inhibition zone of bacterial growth will be closeto zero. MIC can be calculated according to the linear equation Y= a+b X, since the Y is the area of the inhibited zone of bacteria growth a is the intercept, b is the slope and X is the concentration of the antibiotic. The calculations of LOD and LOQ for drug against each bacteria was separately done, the statistical data were as shown in Table No.1.

Accuracy

The accuracy of the proposed method was determined by investigating the recovery percentages of the studied drug at three concentration levels (10, 20 and 40µg/mL) (three replicates of each concentration). The results were shown in Table No.2. The resulted data revealed good accuracy and recovery percentages ranging from 98.86 to 101.01% and from 98.84 to 102.11% for azithromycin against *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively.

Precision

The precision of the developed method was assessed by assay (n = 3) at low (LQC), medium (MQC) and high (HQC) concentration levels (10, 20 and 40/mL) for azithromycin against both *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively (Table No.2). The precision represented as coefficient of variation C.V values that were ranged from 0.29 to 2.14% and from 0.35 to 2.43 for azithromycin against *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively indicating good repeatability and precision. C.V values were satisfactory for application of this method in quality control measurements.

***In-vitro* bioequivalence study**

The proposed method was applied for determination of the recovery percentages of three brands at two concentration levels (10 and 20µg/mL), the results were as shown in Table No.3 and Figure No.3, it was revealed that all the three brands are biologically equivalents science all the dosage forms causing closely related inhibition zones and allowed recovery percentages ranged from 97.27 to 104.82% and from 98.56 to 103.07% against *Klebsiella pneumonia* and *Staphylococcus aureus*; respectively.

Table No.1: Statistical and quantitative parameters for determination of azithromycin by the proposed densitometric method

S.No	Drug against bacteria	LOD µg/mL	LOQ µg/mL	Slope ± SD	Intercept ± SD	Correlation coefficients
1	Azithromycin against *Klebsiella pneumonia*	2.23	6.69	22.32±1.91	545.39±15.06	0.994
2	Azithromycin against *Staphylococcus aureus*	2.22	6.66	38.47±4.54	1171.40±25.93	0.993

Table No.2: The accuracy and precision of the proposed densitometric method for determination of azithromycin

S.No	Drug against bacteria	Concentration (µg/mL)	Recovery [a] % ± SD	C.V
1	Azithromycin against *Klebsiella pneumonia*	10	99.99 ± 0.29	0.29
		20	98.86 ± 1.97	1.99
		40	101.01 ± 2.16	2.14
2	Azithromycin against *Staphylococcus aureus*	10	98.84 ± 0.35	0.35
		20	102.06 ± 1.92	1.88
		40	102.11 ± 2.48	2.43

[a]Average of three determinations

Table No.3: Application of the proposed method for bioequivalence study of three brands containing azithromycin (each tablet contains 500mg)

S.No	Brand	Conc.(µg/mL)	*Klebsiella pneumonia*	*Staphylococcus aureus*
			Recovery *a* %±SD	Recovery *a* %±SD
1	Azicure®	05	99.67 ± 0.87	101.05±1.71
		10	103.52 ± 0.79	101.18±0.86
2	Azithromycin IBN Hayyan®	05	98.83 ± 2.31	99.94 ± 1.29
		10	104.82 ± 1.24	103.07 ± 2.11
3	Maxazi®	05	99.39 ± 1.35	97.27 ± 0.77
		10	101.50 ± 0.62	98.56 ± 1.83

[a]average of three determinations

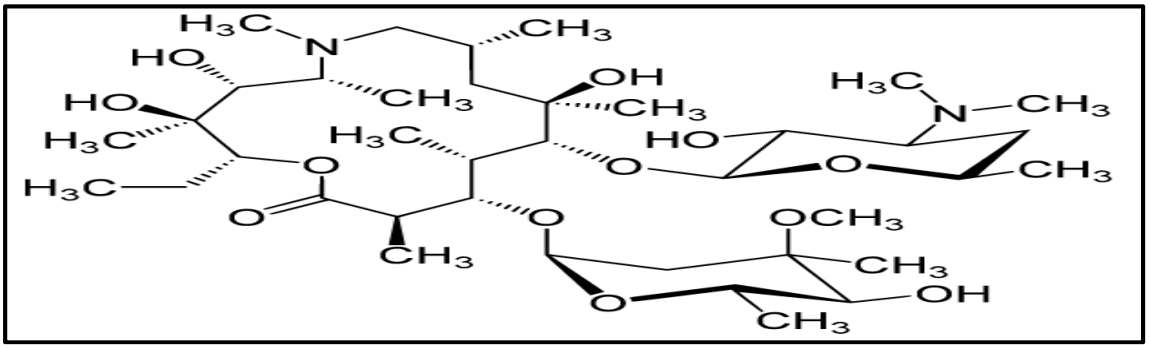

Figure No.1: Chemical structure of azithromycin

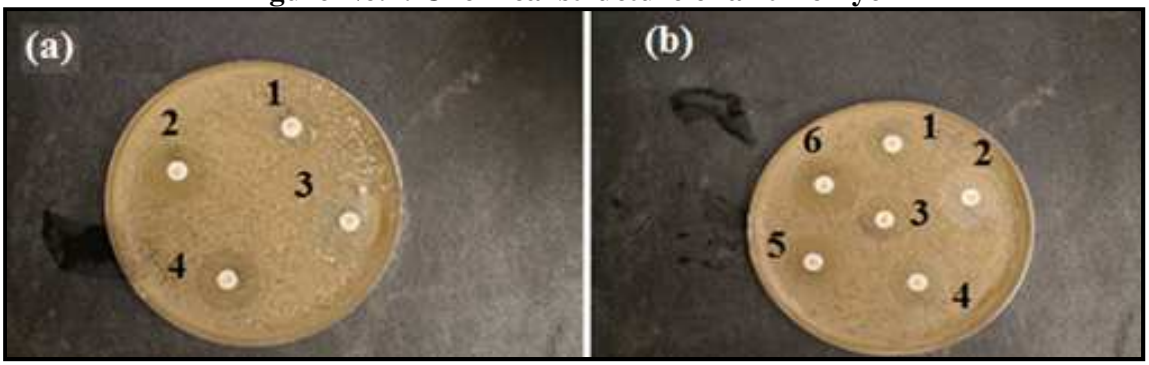

Figure No.2: The antibacterial activity of the studied (a) standard azithromycin (5, 10, 20 and 40µ/mL) and (b) azithromycin in tablets (1, 2, 3, 5; 10µg/mL, 2, 4, 6; 20µg/mL) of the three different brands against *Staphyllococcus aureus* bacteria

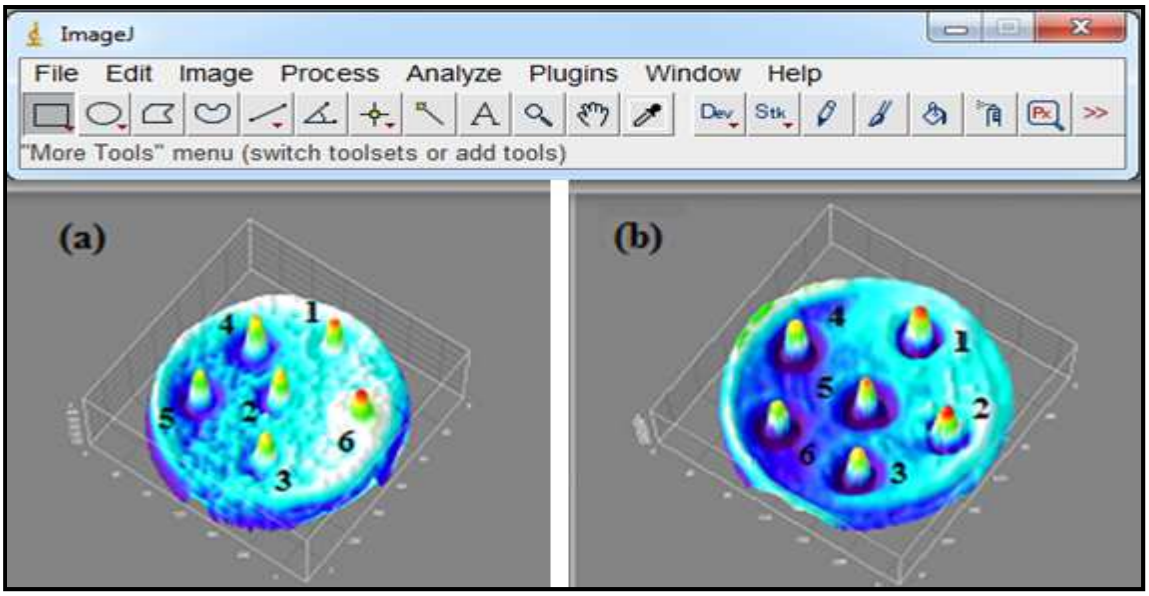

Figure No.3: Interactive 3D surface plot of azithromycin in tablets (1, 2, 3;10µg/mL and 4, 5, 6; 20µg/mL) of the three different brands against (a) *Staphyllococcus aureus* and (b) *Klebcila pneumonia* bacteria obtained by image J software

CONCLUSION

The proposed densitometric technique represents a new and cheapest way for in-vitro bioequivalence study of antibiotics in general and for azithromycin pharmaceutical products (tablets) especially. Low cost of reagents, not need expensive apparatus, rapidity and accuracy of this method makes it applicable in quality control laboratories for determination of azithromycin activity. The accuracy of the method lies in measuring the inhibited zone area exactly by help of Image J software even if this area irregular in shape so improving the accuracy of pharmaceutical analysis and reducing errors of measurements.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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