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Triplicate Design Bioequivalence Studies

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Abstract

The purpose of this study is to apply a triplicate design approach to bioequivalence study of azithromycin following a 500-mg oral dose to 8 subjects, and compare the results with replicate design and the standard average bioequivalence design. This was conducted as a 2-treatment 2-sequence 6-period crossover study. Results from triplicate design expressed lower intra-subject variances in both treatments and also lower subject-by-formulation interaction variance as compared to the replicate design analysis. However, both design conclusions were bioequivalence of both formulations. Results from the average bioequivalence method showed no success to prove bioequivalence of both formulations when performed in each replicate separately. Triplicate designs led to more power to express intra-subject and interaction variances, better than replicate designs, and with minimum number of subjects. In conclusion, triplicate and not replicate designs are suggested to be adopted in future bioequivalence studies, whenever average bioequivalence is not adequate.

Key Words: Bioequivalence, Azithromycin, Replicate design, Triplicate design.

Introduction

Statistical analysis for pharmacokinetic measures, such as area under the curve, using the standard average bioequivalence involves the calculation of a 90% confidence interval for the ratio of the averages (population geometric means) of the measures for the test and reference products. However, the average bioequivalence method does not assess a subject-by-formulation interaction variance, that is, the variation in the average test and refernce difference among individuals. In contrast, the individual BE approach assesses within-subject variability for the test and reference products, as well as the subject-by-formulation interaction (FDA guidance 2001).

Azithromycin is a semi-synthetic antibiotic belonging to the macrolide subgroup of azalides and is similar in structure to erythromycin. Azithromycin exhibits significant intracellular penetration and concentrates within fibroblasts and phagocytes. As a result, tissue levels are significantly higher than plasma concentrations. The half-life of elimination of azithromycin has been reported to be variable and can reach 70 hours, which is partially explained by its extensive tissue uptake and slow tissue release (Schentag, *et al.*, 1999; Peters, *et al.*, 1992; Lalak, *et al.*, 1993; Gladue , *et al.*,1990).

Our own data showed that inter-subject variability of an azithromycin product is as high as 50 %. This can increase the minimum sample size needed for a standard average bioequivalence study to more than 28 subjects, with 80% power. The purpose of this study is to apply a triplicate design bioequivalence study of azithromycin using only 8 subjects, and compare the results with replicate design and the standard average bioequivalence design results.

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Materials and Methods

Drugs and Reagents: Azithromycin 250-mg capsules were purchased from the Jordanian market (Test) and compared with Zithromax®, Batch # 71036070 (Reference). All reagents used were obtained from Sigma Chemical Company, USA.

Subjects: Ten healthy male subjects gave written informed consent to participate in the study. However, subjects 3 and 4 dropped out from the study and were not included in the analysis. The study was approved by the Institutional Review Board of the study site, Ibn-Annafis Hospital. The subjects were within 15 % of their ideal body weight and were judged to be healthy based on medical history, physical examination, complete blood count and serum chemistry. In addition, all subjects were medication free, including over-the-counter drugs, for 7 days prior to the study.

Experimental and Assay Procedure: Following a ten-hour overnight fast, a 500-mg dose of azithromycin capsules was administered orally followed by 240-ml water. Blood samples were collected at $0_{pre\text{-}dose}$, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 18, 24, 48, 72, 96, 120, 144, 168 and 192 hours after dosing. Samples were stored at –20 ^{0}C until analysis. A rapid and sensitive High Performance Liquid Chromatographic (HPLC) method was developed for the determination of azithromycin in plasma using clarithromycin as the internal standard. The procedure involves basification of azithromycin and clarithromycin from 0.5 ml plasma using *tert*-butyl methyl ether as the extraction solvent. The separation of azithromycin was performed using a stainless steel C_8 (4.6 X 100mm) symmetry column with a particle size of 3.5 µm. The mobile phase consisted of 63.5% phosphate buffer and 36.5% acetonitrile. pH was adjusted to 7.40 with concentrated phosphoric acid. The mobile phase was pumped at a flow rate of 1.2ml/min at a constant oven temperature of 35 °C. The effluent was monitored using an electrochemical detector (ECD). Each analysis required no longer than 15 minutes. Quantification was achieved by the measurement of peak-area ratio of the drug and the internal standard. The limit of quantification for azithromycin in plasma was 5 ng/ml. All the samples that were collected after 192 hours after dosing were below the quantification limit.The intra-day and inter-day coefficient of variation (C.V.%) ranged from 3.95 to 7.28% and from 5.42 to 7.61 % respectively at the following concentrations: 30 ng /ml, 150 ng /ml, 300 ng /ml. Relative recovery ranged from 95.25 to 102.05% while the absolute recovery ranged from (92.68 to 101.21)%. Stability test shows that azithromycin is stable in plasma for at least one month when stored at – 20 ± 5 °C.

Replicated Crossover Design: Replicated crossover designs are critical when an individual BE approach is used to allow estimation of within-subject variances for the test (T) and reference (R) measures and the subject-by-formulation interaction variance component. The following six-period, two-sequence, two-formulation design was used: (TRTRTR), (RTRTRT), with a washout period of two weeks.

Data Analysis: The individual pharmacokinetic parameters were calculated by non-compartmental analysis using the software Kinetica2000® (Kinetica 2000 manual, 2000). For the purpose of study design comparison, only the data of area under the curve to last measured time ($AUC_{0\text{-}t}$) was subjected to statistical analysis after logarithmic transformation. Bioequivalence limits were calculated using the method of moments in replicate (replicates 1 and 2) and triplicate (all replicates) designs, and the calculation of a 90% confidence interval for the ratio of the averages in the standard average bioequivalence (FDA Guidance, 2001, Chinchilli, V.M *et al.*, 1996). Detailed presentation of data analysis is beyond the aim of this article. However, within subject

variance formula in replicate designs was adjusted for the triplicate designs and calculated according to:

$$M_k = \sigma^2_{wk} = \left(1/2(n-2) \right) \sum_{j=1}^{j=n_k} \sum_{l=1}^{l=3} \left[K_{jl} - \overline{K}_j \right]^2$$

where, K indicates treatment, j indicates subject on treatment K, l indicates replicate on treatment K for subject j, n_k is the number of subjects on treatment K. K_{jl} is the response of replicate l on treatment k for subject j.

Results and Discussion

Individual data of area under the curve (AUC_{0-t}) for all subjects and replicates were summarized in Table 1.

Table 1. AUC_{0-t} (ng . h/ml) after 500 mg oral dose of azithromycin administered in the triplicate crossover design.

Subject Number	Sequence Number	AUC_{0-t} Replicate 1- Test	AUC_{0-t} Replicate 2- Test	AUC_{0-t} Replicate 3- Test	AUC_{0-t} Replicate 1- Refer.	AUC_{0-t} Replicate 2- Refer.	AUC_{0-t} Replicate 3- Refer.
1	1	3253	4882	6297	3259	5243	5928
2	2	7353	3693	3960	6911	2874	4714
5	1	2576	3414	3219	1620	5070	4374
6	2	4008	1744	3546	4175	2280	2331
7	1	3877	2825	3352	5793	3179	4505
8	2	1979	2474	2015	1873	3582	2106
9	1	5320	3750	3980	1931	1851	3345
10	2	1172	4477	2400	814	6302	2624

Mean values of half-life, maximum drug concentration and the time to reach it, for the test and reference products respectively, were 42.9 hr, 45.6 hr, 319.6 ng/ml, 383.4 ng/ml, 3.02 hr and 2.66 hr. For the purpose of comparing triplicate design with other designs, only AUC_{0-t} parameter was used. Statistical analysis results were summarized in Tables 2-4.

Table 2. Individual bioequivalence analysis results for AUC_{0-t} Parameter of the replicate Design ($\delta = 0.0566$, $\sigma^2_D = -0.30664$)

Parameter	Variance Estimate	Confidence Bound (Hq)	Point Estimate (Eq)	Upper Limit (Uq)
Δ^2	---	0.0401175	0.003203	0.00136
σ^2_I	0.13127	0.4817134	0.131267	0.12281
σ^2_{WT}	0.28997	0.5320595	0.144986	0.14983
σ^2_{WR}	0.58584	-1.1153261	-2.340428	1.50087

$H_{\eta l} = -0.7287$ (Conclusion: Pass, since less than zero).

Table 3. Individual bioequivalence analysis results for AUC_{0-t} Parameter of the triplicate Design ($\delta = 0.02706$, $\sigma^2_D = -0.10311$)

Parameter	Variance Estimate	Confidence Bound (Hq)	Point Estimate (Eq)	Upper Limit (Uq)
Δ^2	---	0.0192879	0.000732	0.00034
σ^2_I	0.08000	0.2916976	0.079488	0.04503
σ^2_{WT}	0.09000	0.1724486	0.046992	0.01574
σ^2_{WR}	0.27000	-0.5163168	-1.083452	0.32164

$H_{\eta l} = -0.3376$ (Conclusion: Pass, since less than zero).

Table 4. Average bioequivalence analysis results for AUC_{0-t} Parameter

	Replicate 1	Replicate 2	Replicate 3
Point Estimate	120.8	92.7	70.8
90% Lower Limit	90.9	71.8	40.4
90% Upper Limit	160.6	119.7	124.1
Conclusion	Fail	Fail	Fail

Intra-subject variability and subject-by-formulation interaction are not accounted for in average bioequivalence studies. This can lead to erroneous decisions in bioequivalence studies such as concluding bioinequivalence for bioequivalent products. Hence the idea of replicate designs came mainly for this purpose (FDA Guidance, 2001). However, replicate designs can give high intra-subject variability and large subject-by-formulation interaction, which in turn can lead to inaccurate decisions in bioequivalence studies by making them easier to pass. On the other hand, triplicate design studies offer the advantage of accounting for intra-subject variability and subject-by-formulation interaction and in a better and more accurate way than replicate designs. This leads to more realistic decisions in bioequivalence studies as compared to other designs.
This can be seen clearly in our azithromycin results. The statistical analysis shown in Table 4 using the average bioequivalence method for each replicate separately led to an erroneous conclusions of bioinequivalency due to the unaccounted for variabilities. However, after applying the replicate design method to the first two replicates as shown in Table 2, results showed high intra-subject variability and large subject-by-formulation interaction, leading to an easy bioequivalence decision. Finally, after applying the triplicate design approach shown in Table 3, we obtained more accurate intra-subject variability and subject-by-formulation interaction estimates leading to a better bioequivalence decision.
In conclusion, authors suggest that triplicate and not replicate designs are to be adopted in future bioequivalence studies, whenever average bioequivalence is not adequate.

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