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The existence of sequence effect in cross-over Bioequivalence trials

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SUMMARY

A generic drug product (T) in order to be approved for marketing authorization a bioequivalence trial is required. In the trial the generic product is compared to the innovator product (R) in terms of the pharmacokinetic parameters AUC and Cmax. The regulatory requirement for bioequivalence is that the 90% confidence intervals for the ratio (T/R) of the generic to innovator product pharmacokinetic parameter averages lies within the limits (80%, 125%). The design of the trial is usually a two-period crossover. This design has the limitation that if the statistical analysis reveal significant sequence effect then the bioequivalence results may be biased and their interpretation is difficult. The sequence effect is confounding with the unequal residual effect and with the formulation by period interaction. Since the existence of the sequence effect questions the quality of the trial, the applicant should provide possible explanations and information on the subjects, the trial conditions, the clinical settings and the assay methodology. An additional statistical analysis on the data from the first period of the trial may support the bioequivalence. If it is proven that the sequence effect is a true effect then the generic may be approved for marketing authorization.

INTRODUCTION

The bioequivalence between a generic (test) and the innovator product (reference) is tested by comparing the relative bioavailabilities of the two products. The bioequivalence is usually demonstrated by the pharmacokinetic parameters: the area under the plasma concentration-time curve from time zero to time infinity (AUC) and the peak plasma concentration (Cmax) which expresses the extent and rate of drug absorption, respectively (1,2).

If bioequivalent is shown between the two products then the generic product is approved for marketing authorization by the regulatory agencies (3).

The design of a bioequivalence trial is usually a two-period balanced cross-over. In this design, the subjects are randomly assigned to two groups of equal size. The subjects in group 1 receive the test product (T) in the first period. Then after a wash-out period they receive the reference (R) product in the second period. In group 2 the subjects receive the products in the reverse order. The design is schematically presented as follows:

	Period 1	Period 1
Sequence 1	T	R
Sequence 2	R	T

This design has the advantage over other designs, e.g. parallel group design, that it requires less number of subjects and the duration of the trial is shorter (2,4).

The statistical evaluation of the trial, e.g. for the parameter AUC, is based on the following linear model:

$$AUC_{ijk} = \mu + subject_{ik} + period_j + product_{(j,k)} + \varepsilon_{ijk},$$

and (1)

$$subject_{ik} = sequence_k + subjects(sequences)_{i(k)},$$

where μ is the overall mean, ε is the error, n_k is the number of subjects in sequence k, i=1-n_k, j=1-2 and k=1-2.

In order to meet the assumptions required for a valid statistical analysis the data are usually log-transformed prior to the analysis (2,5,6).

Then the fit of the model, e.g. for a trial with 24 subjects, produces an analysis of variance (anova) of

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the form shown in Table 2.

The significance of the sequence effect is tested using the subjects within sequences effect (subjects(sequences)) as an error term. The significance of the period and product effects is tested based on the error mean square.

The regulatory requirement for bioequivalence is that the 90% confidence intervals (c.i.) for the ratio (T/R) of the test to reference product pharmacokinetic parameter averages lies within the limits (80%, 125%) (3).

When the statistical analysis reveals significant sequence effect, the bioequivalent results may be bias and the interpretation of the results will be difficult. The applicant should provide explanations about the nature and origin of the effect (1,4,5,7) for the test product to be approved for marketing authorization

However, if a sequence effect is suspected prior to the conduct of the trial, an alternative of the two-period cross-over design should be considered, as a possible parallel group design (2,4,7).

The purpose of this paper is first to investigate the eauses of a sequence effect (1) and second to propose explanations that an applicant should provide to regulatory agencies.

It should be stressed that a not well-conducted trial could be the cause of sequence effect. Thus, although both products may be equivalent, the trial itself will fail to prove it. A statistical analysis on the data from the first period may support the bioequivalence results (9) and to rescue the trial.

METHODS

Confounding of effects

Unfortunately, the two-period cross-over design has the limitation that the true sequence effect is confounded with the differential carry over effect and with the product by period interaction. Confounding means that the sequence effect can not be distinguished from the other mentioned effects and therefore its evaluation requires more information (7,8,9).

The true sequence effect means that the difference between the two sequence groups is attributable only to the difference between the averages of the two groups. If a true sequence effect dose exist the determination of the relative bioavailability T/R is not biased since the anova adjusts the product effect for the sequence effect (5,6).

The residual effect exists when the administration of a drug affects the blood levels of a subsequent administration of another drug. However, the worst case is the existence of an unequal residual effect, i.e. the response in the second period depends on the response in the first period and the variation of the difference depends on which treatment is given in the first period. Then, the validity of the resulted c.i. for T/R is questioned and the test product may not be approved for marketing authorization (4,9).

The significant product by period interaction points out that the difference between the two products depends on the period in which they were administered. Although the existence of such interaction makes the interpretation of the statistical results difficult (9) the product is approved for marketing authorization.

The sequence effect

The actual cause of the sequence effect cannot be determined from the data alone.

For this reason, in addition to the trial data, more information is required on the subjects' condition, the conduct of the trial, the analytical assay techniques, the clinical settings, the weather e.t.c. Thus, before starting the trial it has to be verified that the subjects are healthy and selected appropriately, meeting the inclusion criteria. It has to be tested that the drug is not an endogenous entity and the analytical methodology is well validated (7).

A wash-out period of appropriate length insures that there are no residual effects from a previous administrating period to the next period. If a wash-out period of less than nine half-lives is adopted then is quite possible the administered product of the first period not to be eliminated in the second period. Then there is a residual effect, possibly unequal, and the bioequivalence testing result, the 90% c.i. for T/R, is biased. The trial in this case is unfortunate and the test product is not approved for marketing authorization. Usually, the single-dose trials have an adequate wash-out period to overcome a possible carry-over effect (4,7).

When the randomisation of the subjects to the two sequences is not properly performed, or designed, then it may be possible to detect a sequence effect. Then, it is advised to check the demography of the subjects (age, sex, weight, etc) and to identify which of these parameters are associated with the subject responses. However, the drawback of the bad randomization can be eliminated in the analysis by adjusting for these parameters (4,8).

If the analytical assay procedures are not validated for accuracy and sensitivity, then the resulted plasma concentrations may produce results for AUC and Cmax that give rise to a sequence effect.

When the plasma concentrations in time for each

Table 1. Average and standard deviation of AUC (ng.h/ml) for each product, sequence and period.

	Periods		
	1	2	overall
Products R	490.4±313.1	338.0±215.3	414.2±215.3
T	352.7±185.7	460.7±328.5	406.7±185.7
overall		421.5±185.7	399.3±215.3

Sequences	
TR	RT
345.3±185.7	475.6±313.1

Table 2. The analysis of variance (anova) table for the two-period crossover design. The response is the log-AUC (ng.h/ml).

Source of variation	df	SS	MS=SS/df	F
Subjects	23	3.795		
Sequences	1	1.397	1.397	12.8 *
Subjects within sequences	22	2.397	0.10895	
Period	1	0.01801	0.01801	0.716 n.s.
Products	1	0.00441	0.00441	0.175 n.s.
Error	22	0.554	0.0252	
Total	47	4.37		

* means $P \leq 0.05$, n.s. means $P>0.05$.

subject do not cover a sufficient time period then the statistical analysis, especially for AUC, may produce a significant sequence effect. Thus, it is required blood sampling for a period more than three half-lives to create an adequate profile. Then, the AUC is determined sufficiently.

The sequence effect may be caused by the psychological state of the patients. Their attitudes in the second period may depend on his experience in the first period, especially, when in the first period the reference product was administered. Thus, blindness is recommended for these studies, although it is usually omitted (4,8).

If the patients in the second administration period are exposed to different clinical or weather conditions than in the first period then, it is likely to be a significant product by period interaction. There is variation of the differences between products over the two periods. The true interaction effect is indicated by a plot of the product averages for each period (P_j): TP_1, TP_2, RP_2, RP_2. If the $R_{P2}R_{P2}$ line crosses the $T_{P1}T_{P2}$ line then it is likely to have a true product by period interaction since it is unlikely to have a very large residual effect from T_{P1} (see for example Figure 1). However, the analysis on the transformed (e.g. logarithmic) AUC or Cmax data may remove the effect of the interaction, but this is not always true (9).

A sequence effect may also be caused by the "outlier" values of one or two subjects in one period. In this case these values can be omitted and the bioequivalence testing can be based on the remaining data (10).

When an explanation cannot be given on the existence of the sequence effect then the only way for the applicant to support the bioequivalence of the products is to analyse the data deriving from the first period. Although half of the data are lost, still an analysis can be performed given that the sample size is sufficiently large. Then the design is converted to a parallel group design. The analysis can be done using one-way anova which includes the between product effect and the within product variability which is the error term. This error term is larger than the corresponding error term of the cross-over design without the presence of the sequence effect. Therefore, the c.i. for T/R is expected to be larger than it was anticipated by the cross-over design. For this reason, the number of subjects in the trial should be no less than 24 in order to have the minimum power to detect significant results (4,9).

Example

The relative bioavailability of a test and reference captopril tablets were determined using a single-dose, randomised two-period design with one week wash-out period. Twenty-four healthy subjects participated in the trial. All the patients met the inclusion and exclusion criteria as required by the regulatory agencies. The blood samples were collected for a period up to 12 hours and the sampling time points were kept constant for each subject. The clinical settings were standard and according to the trial protocol. The analytical assay technique was valid and accurate.

The statistical analysis was carried out, for example, only for AUC data after log-transformation. The summary statistics of AUC for each product, sequence and period are shown in Table 1. Since there is apparent difference between the sequence averages there is indication of a sequence effect. This is also indicating by the product by period averages.

The fit of the linear model (1) produced the anova table shown in Table 2. The anova showed that there are no significant period and product effects ($P>0.05$) but there is a significant sequence effect ($P<0.05$). However, the 90% c.i. for T/R is (90%, 106%) which is within the acceptance limits for bioequivalence.

The applicant cannot claim bioequivalence without explaining first the existence of sequence effect. In this trial: i) the washout period was one week (the half-live for captopril is three hours), ii) the subjects were sele-

Table 3. Anova for the AUC (ng.h/ml) data from the first period of the crossover design.

Source of variation	df	SS	MS=SS/df	F
Between products	1	0.7795	0.7795	9.62 *
Within products	22	1.782	0.0810	
Total	23	2.561		

* means $P \leq 0.05$.

cted appropriately and kept under constant conditions in both periods, iii) the clinical and analytical part were sufficient and iv) an appropriate randomization procedure was applied. Then it can be concluded that the sequence effect is a true one.

However, these explanations should be supported by an analysis on the data from the first period only. Then the design is converted into a two-parallel group. The statistical analysis can be performed using one-way anova (see Table 3). The resulted 90% c.i. for T/R is (57%, 85%) which lies outside the acceptance limits. Since this analysis does not support the bioequivalence, the data must be examined closely before the fate of the trial is decided

The closer examination of the results (see Figure 1) reveals that probably there is a true product by period interaction and this caused the existence of the sequence effect. The one week wash-out period excludes the possibility of very large residual effects.

Then, the resulted 90% c.i. for T/R from the crossover design is considered acceptable since anova includes all the possible effects. Therefore, the generic product may be approved for marketing authorization.

DISCUSSION

The approval of a generic product for marketing authorization is usually based on a two-period crossover bioequivalence trial. In this trial the bioavailabilities (AUC and Cmax) of the generic and the innovator product are compared. The statistical analysis involves the anova and the calculation of the 90% c.i. for T/R. If the 90% c.i. lies within the limits (80%, 125%) the two products are considered bioequivalent.

However, there are cases where the statistical analysis reveals significant sequence effect. Then the quality of the trial is questioned. Since the sequence effect is confounded with the unequal residual effect and with the product by period interaction, it makes the interpretation of the results difficult and it may bias them.

Then the applicant should provide possible explanations for the existence of this effect and additional information on the subjects, the trial conditions, the clinical settings and the assay methodology is required.

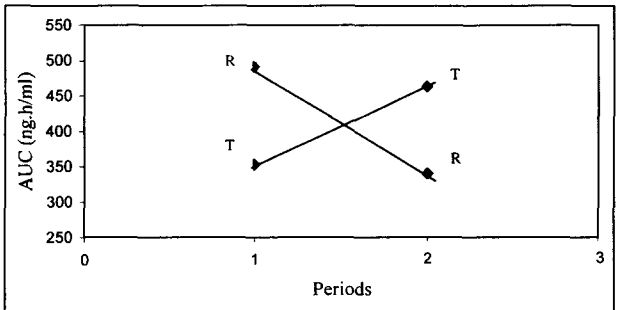

Fig. 1 : Plot of averages for the product by period interaction

If it is proven that the existence of sequence effect is not caused by the above factors and, in addition, the washout period is sufficient and the randomization is properly performed, then there is evidence of a true sequence effect and the bioequivalence results are acceptable by the regulatory authorities. However, it is suggested to conduct an analysis on the data from the first period for supporting the bioequivalence.

When the supportive analysis does not indicate bioequivalence, the data should be examined closely and the existence of a true product by period interaction should also be investigated.

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