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Tutorial

Bioequivalence data analysis

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ABSTRACT

SAS® is commonly used for bioequivalence (BE) data analysis. R is a free and open software for general purpose data analysis, and is less frequently used than SAS® for BE data analysis. This tutorial explains how R can be used for BE data analysis to generate comparable results with SAS®. The main SAS® procedures for BE data analysis are PROC GLM and PROC MIXED, and the corresponding R main packages are "sasLM" and "nlme" respectively. For fixed effects only or balanced data, the SAS® PROC GLM and R "sasLM" provide good estimates; however, for a mixed-effects model with unbalanced data, the SAS® PROC MIXED and R "nlme" are better for providing estimates without bias. The SAS® and R scripts are provided for convenience.

Keywords: SAS®; GLM; MIXED; sasLM; nlme

INTRODUCTION

The SAS® PROC GLM has been used for more than 40 years since 1976, and the SAS® PROC MIXED is a relatively new procedure that has been available since 1992 [1]. The PROC GLM treats all effects as fixed effects for the calculation, while the PROC MIXED is devised to correctly calculate the mixed-effects model including random effects [1]. Because the fixed and random effects are both commonly used in bioequivalence (BE) studies, the MIXED procedure provides a better linear unbiased estimator of the random effects than the GLM for BE analysis [2].

The issue of whether an effect is fixed or random has been discussed in BE studies [2]. Factors that have shown several unique values in experiments or have been chosen intentionally by the investigators are called fixed factors. In BE studies, the period and treatment are typically fixed effects because BE studies are exclusively focused on mean differences and most importantly, the treatment levels resulting from deliberate choice and not from sampling a distribution [3]. On the other hand, many studies use random factors whose levels represent a broader population. For example, the subject is considered as a random factor because it was selected to represent a sample of a population with a probability distribution. Level means and differences for fixed factors can be estimated and tested whereas those for random factors should not be estimated nor tested; only the size of variability (i.e., degree of spread) should be estimated [2].

Table 1. Comparison of the GLM and MIXED methods in Littell et al. [3]

Description	PROC GLM or "sasLM"	PROC MIXED or "nlme"
Estimation	LS method.	ML or REML.
Fixed only vs. mixed	All (fixed or random) effects are initially considered as fixed effects. Variance of random effects is calculated with the estimates of the above.	Variance of random effects is estimated simultaneously with fixed effects.
Biasedness	In case of mixed-effects model with unbalanced data, bias is introduced. This is good only for all fixed effects or mixed model with balanced data.	This gives unbiased estimation with REML, less biased estimation with ML than GLM.

LS, least square; ML, maximum likelihood; REML, restricted maximum likelihood.

The detailed explanation and comparison of the GLM and MIXED analyses in the "SAS® for linear models, 4th ed" [3] are summarized in **Table 1**.

Despite the clear statement in many references that "models that have both fixed and random effects should be analyzed using the PROC MIXED" [1-4], the procedure of GLM rather than MIXED has been mostly used in crossover BE studies (2 × 2) because the two methods produce comparable results when using balanced data [3]. Since the PROC GLM requires balanced data, subjects in a crossover trial who do not provide evaluable data for both the test and reference products (e.g., dropout) should not be included when performing the statistical analysis with the PROC GLM [5]. On the other hand, the PROC MIXED allows the existence of data that are missing at random, there is no need to exclude subjects with missing data [2].

Before the R "sasLM" package became available, it was not feasible to generate the same results as the SAS® PROC GLM in R [6]. The "Anova" function in the "car" package or "drop1" function does not work for BE data that use nested crossover design. However, it is recommended to use the SAS® PROC MIXED or R "nlme" for the significance tests and confidence intervals (CIs).

This tutorial illustrates the comparison between the two procedures (PROC MIXED and PROC GLM) using an example of a simulated dataset for analyzing BE data, and introduces R packages that generated the same results that can be obtained in SAS®. Graphical analysis and sample size determination will be handled in separate tutorials.

METHODS

For the assessment of average BE between formulations in average bioavailability, the usual procedure is as follows:

1) Use log-transformed values for both the areas under the plasma concentration-time curve from 0 to the last measurable concentration (AUC_{last}) and the peak concentrations (C_{max}).
2) Perform ANOVA (e.g., PROC GLM from SAS®) to test the effect of group (or sequence), subject, period, and formulation (or treatment).
3) After the above-mentioned assessment, obtain 90% CIs using the mean squared error of ANOVA, which should be in the range of log(0.80) to log(1.25).

Therefore, the following process of BE analysis is proposed:

1) Use log-transformed values for both AUC_{last} and C_{max}. Data from dropout subjects can be used if non-compartmental analysis can be performed from one or more periods.
2) Test the effects of group (or sequence), subject, period, and formulation (or treatment)

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using the mixed-effects model (e.g., PROC MIXED of SAS® or nlme::lme of R). This test is used for the consideration of the study and not for its invalidation or judgement.

3) After the above test, determine 90% CIs using the estimate for intrasubject variance, which should be in the range of log(0.80) to log(1.25).

Software

SAS® 9.4 and R 4.0.3 were used for the script and results.

Statistical tools for BE analysis

To demonstrate the differences between mixed-effects models through the restricted maximum likelihood estimation versus fixed-effects models, tools originally intended for linear mixed-effects models (SAS® PROC MIXED, "nlme" in R) were compared with those originally intended for fixed-effects models (SAS® GLM, "sasLM" in R). A simulated and unbalanced dataset was used as an example for the BE assessment.

Example dataset

An example dataset is shown in **Fig. 1**.

Note that Subjects 3 and 6 dropped out after period 1 and that their data for period 2 are missing. Subjects with missing observations, such as dropouts should be ignored when using GLM procedure. However, in this example, BE analysis was demonstrated without excluding dropouts to compare the results with the same data. Only C_{max} data are used for demonstration, and AUC data are omitted. If AUC data are available, the relevant AUC column can be added, and the script should be adjusted accordingly.

SAS script

The SAS script of data preparation and check is shown in **Fig. 2**, and those for PROC GLM and PROC MIXED analyses for 2 × 2 BE data are shown in **Figs. 3** and **4**, respectively. For the above analysis, the PROC GLM calculates group (or sequence), subject, period, and formulation (or treatment) as fixed effects, and then considers the subject effect as a random effect after the calculation.

R script

The R script for data preparation is shown in **Fig. 5**, and the scripts equivalent to the SAS PROC GLM and PROC MIXED analyses for 2 × 2 BE data are shown in **Figs. 6** and **7**, respectively. The "nlme" in R and PROC MIXED considers the subject effect as a random effect from the

Subject	Group	Period	Treatment	Cmax
1	TR	1	T	269.3
1	TR	2	R	410.4
2	TR	1	T	120.2
2	TR	2	R	137.3
3	TR	1	T	105.2
4	RT	1	R	90.9
4	RT	2	T	68.9
5	RT	1	R	228.3
5	RT	2	T	301.5
6	RT	1	R	105.3

Figure 1. An example dataset saved as "BEsim.csv."

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```
DATA BESim;
  INFILE '/folders/myfolders/BESim.csv' FIRSTOBS=2 DLM=",";
  INPUT SUBJ $ GRP $ PRD $ TRT $ Cmax;
  LnCmax = LOG(Cmax);

PROC PRINT; RUN;
```

Figure 2. SAS script for data preparation and check.

```
PROC GLM DATA=BEsim;
  CLASS SUBJ GRP PRD TRT;
  MODEL LnCmax = GRP SUBJ(GRP) PRD TRT;
  RANDOM SUBJ(GRP) /TEST;
  LSMEANS TRT / DIFF=CONTROL("R") CL ALPHA=0.1;
  ODS OUTPUT LSMeanDiffCL=LSMD;

DATA LSMD; SET LSMD;
  PE = EXP(DIFFERENCE);
  LL = EXP(LowerCL);
  UL = EXP(UpperCL);
PROC PRINT DATA=LSMD; RUN;
```

Figure 3. SAS script using PROC GLM.

```
PROC MIXED DATA=BEsim;
  CLASS SUBJ GRP PRD TRT;
  MODEL LnCmax = GRP PRD TRT;
  RANDOM SUBJ(GRP);
  ESTIMATE 'T VS R' TRT -1 1 /CL ALPHA=0.1;
  ODS OUTPUT ESTIMATES=ESTIM;
RUN;

DATA ESTIM; SET ESTIM;
  PE = EXP(Estimate);
  LL = EXP(Lower);
  UL = EXP(Upper);
PROC PRINT DATA=ESTIM; RUN;
```

Figure 4. SAS script using PROC MIXED.

```
require(sasLM) # GLM, T3MS, T3test, CIest functions are in sasLM package.
BEdata = read.csv("BEsim4.csv", as.is=TRUE)
BEdata = af(BEdata, c("Subject", "Period", "Group", "Treatment"))
BEdata
```

Figure 5. R script for data preparation.

```
f1 = log(Cmax) ~ Group/Subject + Period + Treatment # R formula for model
GLM(f1, BEdata)                    # ANOVA with I, II, III Sum of Squares
T3MS(f1, BEdata)                   # Table for Type 3 Expected Mean Square
T3test(f1, BEdata, "Group:Subject") # Hypothesis Test using Type 3 Sum of Square
exp(CIest(f1, BEdata, "Treatment", c(-1, 1), 0.10)) # 90% CI of GMR
```

Figure 6. R script equivalent to SAS PROC GLM.
ANOVA, analysis of variance; CI, confidence interval; GMR, geometric mean ratio.

```
require(nlme)
Result = lme(log(Cmax) ~ Group + Period + Treatment, random=~1|Subject,
         data=BEdata)
summary(Result)
VarCorr(Result)              # variances from standard deviations
ci = intervals(Result, 0.90) # 90% CI of log scale difference
exp(ci$fixed["TreatmentT",]) # 90% CI of GMR
```

Figure 7. R script equivalent to SAS PROC MIXED.
CI, confidence interval; GMR, geometric mean ratio.

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beginning of the calculation. In addition, the "nlme" and PROC MIXED can test the effects of group, period, and formulation by F-test or t-test, which is the primary objective of the ANOVA.

The "af" function in the "sasLM" package changes the type of some columns to the factor type. The "af" should be regarded as the abbreviation of "as factor." Note that the difference and CI are estimated in log scales and are reversed using the "exp" function.

One thing to be cautious about is that the subject ID (Subject) should be different for each group. If some of the subject IDs are the same between groups, the random argument of the second line should be changed as follows:

```
Result = lme(log(Cmax) ~ Group + Period + Treatment, random=~1|Group/Subject,
             data=BEdata)
```

The above statement can be used for various crossover designs such as 2 × 4, 6 × 3, and 3 × 3.

RESULTS

The estimate and LSMeans of the GLM and MIXED analyses generate the estimates and the corresponding standard errors of the method means. The "nlme" package of the R software reproduced the same results as "SAS® PROC MIXED" (point estimates 0.8668, 90% CI 0.5565–1.3501 of geometric mean ratio, **Table 2**). Also, considering that the results of the

Table 2. Summary of the SAS and R script results (SAS® PROC MIXED vs. R "nlme")

Description	SAS® PROC MIXED				R "nlme"			
Random effect								
Subject (group)	0.3368				0.33682883			
Residual	0.04701				0.04699731			
Fixed effect								
Source	Numerator Df	Denominator Df	F value	*p*-value	Numerator Df	Denominator Df	(t value)^2	*p*-value
Group	1	4	0.34	0.5903	1	4	0.3414819	0.5903456
Period	1	2	1.06	0.4115	1	2	1.0594267	0.4115420
Treatment	1	2	0.89	0.4458	1	2	0.8867064	0.4457718
Geometric mean ratio								
Point estimate	0.86685				0.8668540			
90% CI	0.55657–1.35012				0.5565718–1.3501148			

CI, confidence interval.

Table 3. Summary of the SAS and R script results (SAS® PROC GLM vs. R "sasLM")

Description	SAS® PROC GLM					R "sasLM"				
ANOVA										
Source	Df	Type III SS	Mean square	F value	*p*-value	Df	Type III SS	Mean square	F value	*p*-value
Group	1	0.163819	0.163819	0.28	0.6236	1	0.16382	0.16382	0.2811	0.6236
Subject (group)	4	2.597930	0.649482	13.28	0.0713	4	2.59793	0.64948	13.2759	0.07127
Period	1	0.038548	0.038548	0.79	0.4684	1	0.03855	0.03855	0.7879	0.46837
Treatment	1	0.038269	0.038269	0.78	0.4698	1	0.03827	0.03827	0.7823	0.46976
Residual	2	0.097844	0.048922	-	-	2	0.09784	0.04892	-	-
Geometric mean ratio										
Point estimate	0.87081					0.8708130				
90% CI	0.55156–1.37486					0.5515549–1.3748684				

ANOVA, analysis of variance; CI, confidence interval.

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"sasLM" package were the same as those produced in SAS® PROC GLM (point estimates 0.8708, 90% CI 0.5515–1.3748 of geometric mean ratio, **Table 3**), the "sasLM" package could be a viable alternative method for calculating the type III sum of squares [6].

By comparing the GLM and MIXED analyses of a BE crossover study with fixed and random effects, the results showed that GLM and MIXED methods produced different F values, mean, and mean differences because the example dataset was unbalanced. The MIXED method ("nlme" in R software) is recommended over the GLM method for analyzing crossover BE studies to appropriately estimate the random between-subject effects and their variance.

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