Protocol A9001500

THE EFFECT OF TIME OF DAY, FASTING, AND MEAL TYPE ON BIOMARKERS OF LIVER INJURY IN HEALTHY SUBJECTS

Statistical Analysis Plan (SAP)

Version: 1.0

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Date: 22-AUG-2017

NOTE: *Italicized* text within this document has been taken verbatim from the Protocol.

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None.

APPENDICES

1. AMENDMENTS FROM PREVIOUS VERSION(S)

None. This first version of the SAP reflects the A9001500 protocol amendment 2 dated 26 July 2017.

2. INTRODUCTION

Primary purpose of the study is to determine the effect of different food regimens on novel and conventional biomarkers of hepatic injury, establish biomarker baseline and determine the intra- and inter-subject variability of such biomarkers under such regimen conditions. In particular, this study will evaluate the concentration of standard and established hepatic injury biomarkers as well as novel biomarkers total and fractionated bile acids, GLDH, and micro RNA-122 during fasting, and after standard, standard high calorie, high fat, and high carbohydrate meals in healthy subjects.

This SAP provides the detailed methodology for summary and statistical analyses of the data collected in study A9001500. This document may modify the plans outlined in the protocol; however, any major modifications of the primary endpoint definition or its analysis will also be reflected in a protocol amendment.

2.1. Study Design

This will be an open label randomized three period four sequence balanced incomplete block design study in healthy volunteers with 12 completers. Each subject will undergo three fed periods of 8 days each which may include a standard, standard high calorie, high calorie high fat or high calorie high carbohydrate meals. At the end of each 8-day fed period, there will be a fast until 1pm on Day 9 followed by an 11-day washout between periods.

Table 1. Balanced Incomplete Block Design With Proposed Treatment Sequences

Sequences	***Sequence Label***	***Regimen***		
		Period 1	***Period 2***	***Period 3***
1	*ABC*	*A*	*B*	*C*
2	*BAD*	*B*	*A*	*D*
3	*CDA*	*C*	*D*	*A*
4	*DCB*	*D*	*C*	*B*

Meal Treatments:
A: Standard diet; B: High calorie standard diet; C: High fat high calorie diet; D: High carbohydrate high calorie diet

Each subject will have five visits: one clinic visit for screening, three inpatient visit periods, and one follow-up telephone call. The maximum participation is expected to be less than 88 days, including screening. Screening activities will occur at the PCRU and the maximum interval between screen and period 1 will be no more than 28 days. On Day -1 of each period subjects will be admitted to the PCRU for an overnight stay to ensure dietary adherence prior to Day 1.

2.2. Study Objectives

Primary Objectives:

- *To determine the effect of diet type on concentration of standard hepatic injury biomarkers over time and provide their intra- and inter-subject variability estimates.*
- *To determine the effect of diet type on concentration of novel hepatic injury biomarkers, including total and fractionated bile acids, GLDH, and micro RNA-122 over time and provide their intra- and inter-subject variability estimates.*
- *Establish biomarker baseline for standard and novel hepatic injury biomarkers.*

Secondary Objectives:

- *To determine the effect of diet type on fasting lipid panel over time and provide their intra- and inter-subject variability estimates.*
- *To determine the response of, and differences in total and fractionated bile acids and triglycerides under fasting and fed state after a standard, standard high calorie, high fat, and high carbohydrate meals.*
- *To evaluate the association between standard lab values (ie bilirubin, ALT, AST, GGT, ALKP, total cholesterol, LDL, HDL, triglycerides and CK) and meal type.*

Tertiary/Exploratory Objectives:

- *To determine the effect of diet type on weighted-mean daily triglycerides over time.*
- *To evaluate the association between total and fractionated bile acid levels and triglycerides in fed state.*
- *To evaluate the association between changes in fasting total and fractionated bile acid concentrations and, fasting liver function tests and fasting lipid panel biomarkers on Day 8.*
- *To collect banked biospecimens for exploratory research, unless prohibited by local regulations or ethics committee decision.*
- *Explore the use of microRNA profiling.*
- *To evaluate the association between genotype and total and fractionated bile acid concentration.*

3. INTERIM ANALYSES, FINAL ANALYSES AND UNBLINDING

No formal interim analysis will be conducted for this study. Final analysis will follow the official database release.

4. HYPOTHESES AND DECISION RULES

4.1. Statistical Hypotheses

No hypotheses are required.

4.2. Statistical Decision Rules

No decision rules are required.

5. ANALYSIS SETS

5.1. Full Analysis Set

Not applicable.

5.2. Pharmacokinetic Analysis Set

Not applicable.

5.3. Pharmacodynamic Analysis Set

The PD analysis set is defined as all enrolled subjects who receive at least one diet regimen and who have at least 1 of the endpoints/biomarkers of interest.

5.4. Safety Analysis Set

All subjects who receive at least one diet regimen will be included in the safety analyses and listings.

5.5. Other Analysis Sets

None.

5.6. Treatment Misallocations

Subjects will not receive any investigational product in this study, study treatments refer to the diet regimens. For all outputs produced, the following labels and ordering will be used corresponding with the following:

- Standard diet
- High calorie standard diet
- High fat high calorie diet
- High carbohydrate high calorie diet

All analyses will be performed on an "as-treated" basis and will not include data from subjects who are randomized but not treated.

5.7. Protocol Deviations

A full list of protocol deviations will be compiled and reviewed to identify major and minor deviations prior to database closure. Any major protocol deviations may result in sensitivity analyses being produced which will be fully documented.

5.7.1. Deviations Assessed Prior to Randomization

At screening, the investigator will assess subjects against the inclusion and exclusion criteria as set out in Sections 4.1 and 4.2 of the protocol.

5.7.2. Deviations Assessed Post-Randomization

Any significant deviation from the protocol will be reviewed prior to database closure and a decision taken regarding evaluation for each analysis population.

6. ENDPOINTS AND COVARIATES

6.1. Efficacy Endpoint(s)

None.

6.2. Safety Endpoints

In this section, the safety endpoints that will be measured during the study are detailed. Where applicable, details of the endpoints to be derived and definition of baseline are also provided.

The following data are considered in standard safety summaries (see protocol for collection days and list of parameters):

- adverse events,
- laboratory data,
- vital signs data,
- weight.

6.2.1. Adverse Events

Any events occurring following start of treatment or increasing in severity will be counted as treatment emergent.

Events that occur in a non-treatment period (for example, washout or follow-up) will be counted as treatment emergent and attributed to the previous diet regimen taken.

6.2.2. Laboratory Safety Tests

Safety laboratory tests will be performed as described in the protocol. Baseline will be defined as the measurement on Day -1 in each period.

6.2.3. Vital Signs

Single supine blood pressure and pulse rate will be taken at screening, Day 1 (pre-breakfast) and on Day 10.

6.2.4. Other Safety Data

Additional safety data will be collected as described in the protocol and will be listed if collected in the sponsor's database. Baseline for weight will be defined as the measurement on Day -1 in each period.

6.3. Pharmacokinetic Endpoints

None.

6.4. Pharmacodynamic Endpoints

Serum liver function tests and novel hepatic injury biomarkers will be taken according to the Schedule of Activities given in the protocol. For a given parameter and each period, baseline is the Day 1 0H fasting value.

There are nine fractionated bile acids: TCA, CA, GCA, CDCA, GCDCA, TCDCA, TDCA, DCA and GDCA.

For all endpoints the percent change from baseline = 100 *((timepoint – baseline) / baseline).

Primary Endpoints

- Percent *change from baseline in standard hepatic injury biomarkers including ALT, AST, ALKP, GGT, and bilirubin concentration over time*.

- Percent *change in C_{max}, T_{max}, and AUC during the post meals period (8am-1pm, 1pm-6pm, and 6pm-8am) for total and fractionated bile acids.*

For each subject in every period and every post meal period 8am-1pm, 1pm-6pm and 6pm-8am for total and fractionated bile acids the following three endpoints will be derived using absolute values:

- C_{max} defined as the maximum bile acid value
- T_{max} defined as the time for C_{max}
- AUC which is defined as the area under the bile acid profile from start time to the stop time

$AUC_{8\ am\text{-}1\ pm}$, $AUC_{1\ pm\text{-}6\ pm}$, and $AUC_{6\ pm\text{-}8\ am}$ will be calculated using the linear trapezoidal rule in any subject with at least the first, last, and at least 5 values within the given interval available. The nominal time post dose will be used. $AUC_{8\ am\text{-}1\ pm}$ will use the 0 to 5 hours timepoints after breakfast; $AUC_{1pm\text{-}6pm}$ will use the 5 to 10 hours timepoints after breakfast; $AUC_{6\ pm\text{-}8\ am}$ will use the 10 to 0 hours (the next day) timepoints after breakfast.

- Percent *change from baseline in fasting total and fractionated bile acids, GLDH, and micro-RNA 122 concentration over time.*

- *Concentration at baseline on Day 1 at 0H*

This will be the baseline concentration in Period 1 only.

Secondary Endpoints

- *Percent change from baseline in total cholesterol, LDL, HDL, and triglycerides over time.*

- *Percent change in AUC for total and fractionated bile acids and triglycerides from 8am-1pm assessed on Day 8 and 9.*

- *Correlation of regimen on Day 8 and Day 9 with fasting concentration of standard lab values.*

Tertiary/Exploratory Endpoints

- Percent *change in AUC0-24/24 triglycerides during the post meals periods.*

- *Correlation of AUCs (ie, $AUC_{8am-1pm}$, $AUC_{1pm-6pm}$, and $AUC_{6pm-8am}$) for total and fractionated bile acid and triglycerides AUCs (ie $AUC_{8am-1pm}$, $AUC_{1pm-6pm}$, and $AUC_{6pm-8am}$) assessed in the fed states on Day 8.*

- *Correlation of fasting total and fractionated bile acid concentration and fasting bilirubin, ALT, AST, GGT, ALKP, total cholesterol, LDL, HDL, triglycerides, and CK in the fed states after a standard, standard high calorie, high fat and high carbohydrate meal.*

- *Collection of banked biospecimens unless prohibited by local regulations or ethics committee decision. Additional information on collection and potential use is provided in the Banked Biospecimens section.*

- *Develop data on the exploratory biomarker microRNA.*

- *Correlation of genotype with serum total and fractionated bile acid concentration in the fasted and fed states after a standard, standard high calorie, high fat and high carbohydrate meal.*

6.5. Covariates

None.

7. HANDLING OF MISSING VALUES

For the analysis of safety endpoints, the sponsor data standard rules for imputation will be applied. For the novel hepatic injury biomarkers, in all data presentations (except listings), concentrations below the limit of quantification (BLQ) will be set to LLOQ/2. In listings,

BLQ values will be reported as "<LLQ", where LLQ will be replaced with the value for the lower limit of quantification.

8. STATISTICAL METHODOLOGY AND STATISTICAL ANALYSES

8.1. Statistical Methods

As this is an exploratory study, no formal hypothesis testing will be performed.

8.2. Statistical Analyses

The mixed effects model will be implemented using SAS Proc Mixed, with REML estimation method and Kenward-Roger degrees of freedom algorithm. An unstructured covariance matrix will be used to estimate the variances and covariance within subject across time points. If convergence is not obtained or model fit is not adequate then other covariance structures will be investigated as necessary.

Where applicable, the baseline will be fitted as two terms - the average of the three baseline scores (one for each treatment period) for each subject (between-subject baseline) and the difference between the individual period baseline scores and the average for each subject (within-subject baseline). All statistical analyses will only compare each regimen against standard diet.

Example SAS codes are provided in Section 10.

The protocol refers to microRNA as a biomarker of interest. The SAP will focus on miRNA-122 as this may be associated with the liver and so is of specific interest as a useful drug development liver injury biomarker. Any reference to microRNA in the protocol has been replaced with miRNA-122 in this SAP.

Standard summaries will include N, arithmetic mean, median, %CV, standard deviation, minimum, maximum. Percentage changes from baseline will be summarized with the following statistics: N, arithmetic mean, median, standard deviation, %CV, range, geometric mean.

8.2.1. Pharmacodynamic Analysis

8.2.1.1. Primary Analysis

Absolute and percent changes from baseline for standard and novel hepatic injury markers (ALT, AST, ALKP, GGT, bilirubin, total and fractionated bile acids, GLDH, and microRNA (ie, miRNA-122)) will be summarized descriptively by day and regimen. For a given parameter and each period, baseline is the Day 1 0H fasting value. A median profile plot of the percent changes from baseline will be created over time for each biomarker.

Natural log transformed relative change from baseline will be analyzed with a mixed model repeated measures approach using the 0 h measurements obtained from Day 2 through to Day 10. The model will include regimen, day, period, log baseline, sequence and regimen by day as fixed factors and subject nested in sequence as a random effect. Baseline will be included as a between- and within- subject covariate. *LS-means estimates and their 90%*

confidence intervals will be obtained for each regimen averaged across time points and for each day. Differences (and 90% confidence intervals) between LS means will be obtained comparing regimens. All LS means and differences (including CI's) will be back transformed to give geometric LS means and ratios of geometric LS means.

The percent change is then calculated as follows:

Percent change = 100* (RC - 1)

where RC is either the adjusted geometric LS mean estimate or the ratio of adjusted geometric means coming from the statistical model. The corresponding 90% CIs will be calculated as well. A table for the inter- and intra-subject variability from the statistical model will be produced.

Plots of LS-mean estimates and differences will also be produced. No adjustments for multiple comparisons will be used.

For each post meal period 8am-1pm, 1pm-6pm and 6pm-8am for total and fractionated bile acids C_{max}, T_{max} and AUC will summarized by day and regimen for each post meal period.

The following boxplots will be produced and paged by biomarker:

- Three panels per AUC with diet regimen on the x-axis and one box per day
- Three panels per day with diet regimen on the x-axis and one box per AUC

Individual plots will also be produced with 1 panel per subject - each panel will have 1 line per regimen and the x-axis will be hours post breakfast. Each AUC will be presented in a separate output and will be paged by biomarker.

Natural log transformed AUC for each post meal period ($AUC_{8am-1pm}$, $AUC_{1pm-6pm}$ and $AUC_{6pm-8am}$) will be analyzed with a mixed model repeated measures approach. The model will include regimen, day, period, sequence and regimen by day as fixed factors and subject within sequence as a random effect. LS-means estimates and their 90% confidence intervals will be obtained for each regimen and day. Differences (and 90% confidence intervals) between LS means will be obtained comparing regimens for each day. All LS means and differences (including CI's) will be back transformed to give geometric LS means and ratios of geometric LS means. Plots of LS-mean estimates and differences will also be produced.

8.2.1.2. Secondary Analysis

Absolute and percent changes from baseline for lipid panel (total cholesterol, LDL, HDL and triglycerides) will be summarized descriptively by day and regimen. For a given parameter and each period, baseline is the Day 1 0H fasting value. A median profile plot of the percent changes from baseline will be created over time for each biomarker.

Natural log transformed relative change from baseline will be analyzed with a mixed model repeated measures approach using the 0 h measurements obtained from Day 2 through to Day 10. The model will include regimen, day, period, log baseline, sequence and regimen day as fixed factors and subject nested in sequence as a random effect. Baseline will be included as a between- and within- subject covariate. *LS-means estimates and their 90% confidence intervals will be obtained for each regimen averaged across time points and for each day. Differences (and 90% confidence intervals) between LS means will be obtained comparing regimens. All LS means and differences (including CI's) will be back transformed to give geometric LS means and ratios of geometric LS means.*

The percent change is then calculated as follows:

Percent change = 100* (RC - 1)

where RC is either the adjusted geometric LS mean estimate or the ratio of adjusted geometric means coming from the statistical model. The corresponding 90% CIs will be calculated as well.

Plots of LS-mean estimates and differences will also be produced. No adjustments for multiple comparisons will be used.

In order to evaluate the effect of the different diets on total and fractionated bile acids, and triglycerides during the fasted (ie, Day 9) on fed state (ie, Day 8), individual Fed $AUC_{8am-1pm}$/Fasted $AUC_{8am-1pm}$ ratio will be calculated and summarized by regimen.

The natural log transformed ratio will be analyzed with a mixed model repeated measures approach. The model will include regimen, period, and sequence as fixed factors and subject within sequence as a random effect. LS-means estimates and their 90% confidence intervals will be obtained for each regimen. Differences (and 90% confidence intervals) between LS means will be obtained comparing regimens. All LS means and differences (including CI's) will be back transformed to give geometric LS means and ratios of geometric LS means. Plots of LS-means estimates and differences will also be produced.

The association between regimens and standard lab values (ie bilirubin, ALT, AST, GGT, ALKP, total cholesterol, LDL, HDL, triglycerides and CK) will be analyzed using a linear model, with natural log transformed relative change from baseline for standard lab values as the response variable and regimen and day as fixed factors. Two-way interaction among the fixed factors may also be evaluated. Standard diet, high calorie standard diet, high fat high calorie diet, and high carbohydrate high calorie diet will be coded 1, 2, 3 and 4 respectively.

Estimates of the slope and intercept, together with their precision (90% confidence interval [CI]), and the coefficient of determination (ie, R-squared and adj-R-squared) will be obtained from the model.

8.2.1.3. Tertiary/Exploratory Analysis

Percent change in AUC_{0-24}/24 triglycerides during the post meals periods will be summarized descriptively by day and regimen.

Natural log transformed $AUC_{0-24}/24$ will be analyzed with a mixed model repeated measures approach. The model will include regimen, day, period, sequence and regimen by day as fixed factors and subject within sequence as a random effect. LS means estimates and their 90% confidence intervals will be obtained for each regimen and day. Differences (and 90% confidence intervals) between LS means will be obtained comparing regimens for each day. All LS means and differences (including CI's) will be back transformed to give geometric LS means and ratios of geometric LS means. Plots of LS-mean estimates and differences will also be produced.

The correlation of AUCs (ie, $AUC_{8am-1pm}$, $AUC_{1pm-6pm}$, and $AUC_{6pm-8am}$) for total and fractionated bile acid and triglycerides AUCs (ie, $AUC_{8am-1pm}$, $AUC_{1pm-6pm}$, and $AUC_{1pm-6pm}$) assessed in the fed states on Day 8 will be analyzed using a linear model, with natural log transformed AUCs for total and fractionated bile acid and triglyceride values as the response variable and regimen as fixed factors. Standard diet, high calorie standard diet, high fat high calorie diet, and high carbohydrate high calorie diet will be coded 1, 2, 3 and 4 respectively. Estimates of the slope and intercept, together with their precision (90% confidence interval [CI]), and the coefficient of determination (ie R-squared and adj-R-squared) will be obtained from the model.

The correlation of fasting total and fractionated bile acid concentration and fasting bilirubin, ALT, AST, GGT, ALKP, total cholesterol, LDL, HDL, triglycerides, and CK in the fed states (ie, steady state on Day 8) will be calculated using pearson correlation coefficients and paged by diet regimen.

Exploring the use of MicroRNA profiling is considered exploratory and may not be included in the CSR. Post-hoc analyses may be produced and included in a separate report.

The correlation of genotype with serum total and fractionated bile acid concentration in the fasted and fed states is considered exploratory and may not be included in the CSR. Post-hoc analyses may be produced and included in a separate report.

8.3. Safety Analysis

A set of summary tables split by diet regimen will be produced to evaluate the toleration of the diet regimen.

No formal analyses are planned for safety data. The safety and other endpoints detailed in Section 6.2 will be listed and summarized in accordance with sponsor reporting standards, where the resulting data presentations will consist of subjects from the safety analysis set.

8.3.1. Treatment and Disposition of Subjects

Subject evaluation groups will show end of study subject disposition. Frequency counts will be supplied for subject discontinuation(s) by treatment.

Data will be reported in accordance with the sponsor reporting standards.

8.3.2. Demographic and Clinical Examination Data

A breakdown of demographic data will be provided for age, race, weight, body mass index and height. Each will be summarized by sex at birth and 'All Subjects' in accordance with the sponsor reporting standards.

8.3.3. Discontinuation(s)

Subject discontinuations, temporary discontinuations due to adverse events will be detailed and summarized by treatment.

Data will be reported in accordance with the sponsor reporting standards.

8.3.4. Adverse Events

Adverse events will be reported in accordance with the sponsor reporting standards.

8.3.5. Laboratory Data

Laboratory data will be listed and summarized by treatment in accordance with the sponsor reporting standards. Baseline is as defined in Section 6.2.2.

8.3.6. Vital Signs Data

Supine systolic/diastolic blood pressure and pulse rate will be listed.

8.3.7. Other Safety Data

Absolute values and changes from baseline in weight will be summarized by treatment and day. Baseline is as defined in Section 6.2.2. Weight data will also be listed.

8.3.8. Concomitant Treatments

All concomitant medication(s) as well as non-drug treatment(s) will be provided in the listings.

8.3.9. Screening and Other Special Purpose Data

Prior medication(s) and non-drug treatment(s), serum FSH concentrations, urine drug screen and ECG will be obtained at Screening.

These data will not be brought in-house, and therefore will not be listed.

9. REFERENCES

10. APPENDICES

Appendix 1. SAS CODE FOR ANALYSES

Example MMRM Analysis Code:

```
proc mixed data = biomarker;
  class regimen day period sequence;
  model log_resp = regimen day period log_base1 log_base2 sequence regimen*day / solution ddfm = kr residual ;
  random subject(sequence);
  lsmeans regimen regimen*day / diff cl  alpha = 0.1;
  repeated day / subject = subject*period type = un r rcorr;
run;
quit;
```

where base1 is the average baseline for each subject, base2 is the period adjusted baseline

Example Correlation Analysis Code:

```
proc reg data = biomarker;
  ods select fitplot;
  model logauc = regimen day clb alpha=0.1;
  ods output ParameterEstimates = param1;
  ods output FitStatistics = fit1;
  ods output ANOVA = reg1;
run;
```