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Statistical Analysis Plan

SCR-010: Clinical Study to Investigate the Urinary Excretion of N-nitrosodimethylamine (NDMA) after Ranitidine Administration

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Contents

Abbreviations and definitions

AE	Adverse event
ANOVA	Analysis of variance
$AUC_{0\text{-}inf}$	Area under the plasma concentration time curve from time 0 extrapolated to infinity
C_{last}	Last quantifiable concentration
C_{max}	Maximum observed plasma concentration
DMA	Dimethylamine
ECG	Electrocardiogram
eCFRs	Electronic case report forms
inf	Infinity
K_{el}	Elimination rate constant
NDMA	N-nitrosodimethylamine
PK	Pharmacokinetic
SAP	Statistical analysis plan
SD	Standard deviation
$t_{1/2}$	Terminal half-life
TEAE	Treatment-emergent adverse event
Tmax	Time of maximum concentration (C_{max})

Change Log

Version	Section	Changes
2.0	General (Rationale)	NDMA analyses will be based on arithmetic means instead of geometric means. Blinded review of the bioanalytical data revealed that a large number of urine and plasma samples for NDMA were below the lower limit of quantification (LLOQ). For multiple subjects, all samples from 1 or more study periods were below the LLOQ. As zero values cannot be log-transformed for geometric mean analyses, these periods would have been removed from analysis. Furthermore, as analyses are paired, these subjects would have been removed from analyses. Utilizing arithmetic mean will allow data from all subjects and all periods to be used in the planned analysis. Ranitidine and DMA analyses will remain the same (i.e. utilizing geometric means).
	7.3.1	**Analysis of urine concentrations** Analysis of NDMA urine 24-hr excretion will be performed using arithmetic means instead of geometric means.
	7.3.2	**Plasma pharmacokinetics** Descriptive statistics for plasma PK parameters of NDMA will be calculated using arithmetic means.

1. Introduction

This document outlines the proposed statistical methods for data analysis of data collected from Protocol 'SCR-010 Clinical study to investigate the urinary excretion of N-nitrosodimethylamine (NDMA) after ranitidine administration'.

2. Objectives

The following analysis plan provides the framework for the summarization of the data from this study. The analysis plan may change due to unforeseen circumstances but will be finalized prior to database lock. Any changes made after the database lock will be documented and justified in the clinical study report.

2.1 Primary Objective

The primary objective is to evaluate 24-hour urinary excretion of N-nitrosodimethylamine (NDMA) after oral administration of ranitidine compared to placebo.

2.2 Exploratory Objectives

Exploratory objectives include the following:

- To evaluate plasma ranitidine, NDMA, and dimethylamine (DMA) after oral administration of ranitidine compared to placebo.
- To evaluate urinary excretion amounts over 24-hours of ranitidine and DMA after oral administration of ranitidine compared to placebo.
- To evaluate 24-hour urinary excretion and plasma concentration of NDMA and DMA with administration of high nitrite/NDMA meals compared to low nitrite/NDMA meals.

3. Study Overview

3.1 Study Design

This is a randomized, placebo-controlled, single-dose, 4-period crossover study with 18 healthy subjects. Subjects will receive 4 treatments over the 4 study periods (see Table 1). Each subject will be randomized to 1 of 4 treatment sequences (i.e., ABCD, ABDC, BACD, or BADC). The treatment will consist of oral administration of either a single dose of ranitidine (300 mg) or placebo administered at time zero on each of 4 different treatment days. All subjects will be provided low nitrite/NDMA meals for the first 2 periods of the study and high nitrite/NDMA meals for the last two periods of the study. Subjects will check in on Day -2 for baseline assessments and will complete each 2-day period over the next 8 days, followed by checkout (see Table 2).

Two different full day menus of low nitrite/NDMA and high nitrite/NDMA meals have been developed. Meals will be identical for treatment periods 1 and 2(low nitrite/NDMA meals) of the study and a separate set of identical low nitrite/NDMA meals will be served on the washout days prior to treatment. Likewise, meals will be identical for treatment periods 3 and 4(high nitrite/NDMA meals) and a separate set of identical high nitrite/NDMA meals will be served on the washout days prior to treatment days. The last meal on Day -1, Day 2, Day 4, and Day 6 should be administered at approximately 6 PM to permit at least 12-hour fasting prior to dosing. Subjects will be provided with distilled water to drink throughout the study.

On study treatment days, the first meal will be provided at the time of dosing. Subjects will be instructed to swallow the medication with approximately 250 mL of room temperature distilled water and begin eating within two minutes after dosing. Subjects are required to eat each meal in its entirety during the study. If the meal is not finished, the reason should be recorded, along with what was not eaten, and a picture of the remaining food should be taken.

Urine samples will be collected using separate collection containers over 24 h. Collection times will occur at 0 (pre-dose), 3, 6, 9, 12, 15, and 24 h on study dosing days. Subjects will be instructed to void their bladder at each collection time and total weight of the sample will be recorded. If a subject must void their bladder at an unscheduled time (highly discouraged), the unscheduled voids will be collected, and total weight of the unscheduled voiding will be recorded. The unscheduled voiding sample will be treated, analytically analyzed, and reported as part of scheduled sample collection for determining cumulative amounts of NDMA, ranitidine, and DMA excreted over 24 h. Plasma samples for NDMA, DMA, and ranitidine analysis will be collected at 0 (pre-dose), 0.5, 1, 1.5, 2, 3, 4, 5, 6, 9, 11, 14, and 24 h post-dose on study dosing days.

The study will be conducted at one center in the United States (Spaulding Clinical Research unit in West Bend, Wisconsin). Each treatment group will attempt to include equal representation of male and female subjects.

Table 1: Study Treatments

Treatment	Drug
A	Ranitidine (300 mg) + low nitrite/NDMA meals
B	Placebo + low nitrite/NDMA meals
C	Ranitidine (300 mg) + high nitrite/NDMA meals
D	Placebo + high nitrite/NDMA meals

Table 2: Study Design

Day -2	Day -1	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Check-in	Washout	Period 1	Washout	Period 2	Washout	Period 3	Washout	Period 4	Check-out

3.2 Sample Size

Eighteen healthy participants are planned for enrollment. It is estimated that a sample size of 14 completing subjects will provide at least 90% power at a 0.05 significance level to demonstrate an increase in 24-h urinary excretion of NDMA with ranitidine compared to placebo for a 1-sided log-transformed paired comparison. This assumes that geometric mean ratio between 24-h urinary NDMA with ranitidine and placebo is 2 and that the coefficient of variability is 100%. Enrolling 18 subjects allows for up to 20% dropouts.

Sample size calculations for the study are based on results from Zeng and Mitch (2016), which showed a 400-fold increase in NDMA for 150 mg ranitidine compared to placebo (47,600 ng versus 110 ng, respectively). The observed 24-h urinary excretion of NDMA with placebo for this and other studies ranged between 11-380 ng. It is not known if 24-h urinary excretion of NDMA with placebo is higher than what has been historically observed or if the fold-increase observed in Zeng and Mitch with ranitidine would be observed in a study controlling for meals with a randomized crossover design. The study sample size reflects assumptions that the overall fold-increase may be substantially lower but is still powered to detect an increase with assumptions as stated above.

4. Study Endpoints

4.1 Primary Endpoints

The primary endpoint of this study is 24-h urinary NDMA excretion.

4.2 Exploratory Endpoints

The exploratory endpoints of this study are:

1. AUC from time 0 extrapolated to infinity ($AUC_{0\text{-}inf}$) of plasma ranitidine, NDMA and DMA
2. Cumulative ranitidine and DMA amount excreted in urine over 24 h after ranitidine administration

5. Analysis Populations

The PK population will include all subjects who receive study drug and have at least one on-treatment sample. The safety population will include all subjects who receive at least 1 dose of any of the study drugs. The analysis population will include all subjects in the PK population with sufficient data to calculate PK parameters from at least two study periods of the same meal

type (i.e., low nitrite/NDMA or high nitrite/NDMA). Treatment in all analysis populations will be assigned based upon the treatment which the subjects actually received.

6. Data Screening and Acceptance

6.1 Handling of Missing and Incomplete Data

The following imputation of missing values will be done:

- PK measurements (urine or plasma) below the quantification limits will be considered equal to zero for all analyses.
- Missing PK data (e.g., skipped plasma sample collected or uncollected urine voids) will not be imputed.

7. General Statistical Considerations

All data will be presented in data listings. Data from subjects excluded from an analysis population will be presented in the data listings, but not included in the calculation of summary statistics.

7.1 Subject Disposition

The number of subjects who enroll in the study and the number and percentage of subjects who complete each assessment will be presented. The frequency and percentage of subjects who withdraw or discontinue from the study and the reason for withdrawal or discontinuation will be summarized.

7.2 Demographic and Baseline Characteristics

Continuous demographic and baseline characteristic variables (age, height, weight, and body mass index) will be summarized overall and by treatment using descriptive statistics (number of subjects, mean, standard deviation [SD], median, minimum, and maximum). The number and percentage of subjects in each class of categorical demographic and baseline characteristic variables will also be summarized.

7.3 Pharmacokinetic Analyses

7.3.1 Urine Pharmacokinetics

The primary endpoint is a comparison of 24-h urinary NDMA excretion for ranitidine versus placebo when administered with either low nitrite/NDMA meals (comparison between treatments A and B, respectively) or high nitrite/NDMA meals (comparison between treatments C and D, respectively). No adjustment for multiplicity is planned as these comparisons are considered two separate studies for purposes of our overall design. As an exploratory analysis, 24-h urinary DMA for ranitidine versus placebo will likewise be compared between treatments A and B (low nitrite/NDMA meals) and treatments C and D (high nitrite/NDMA meals). 24-hr urinary excretion of ranitidine (exploratory) will be calculated for treatments A and C, though no

comparisons are planned. Finally, as an exploratory measure, the two placebo-treated treatments (treatment D versus B) will be compared to determine if meals comprised of foods traditionally identified as being higher in nitrites/NDMA result in a higher 24-hr urinary excretion of NDMA or DMA compared to meals identified as being lower in nitrites/NDMA.

The cumulative amount of NDMA (primary), DMA (exploratory), and ranitidine (exploratory) excreted in urine over 24-h for placebo and ranitidine administration will be calculated for each subject. All urine voids over a 24-h period will be collected. Each separate void will be weighed in a tarred contained. The volume of the void will be determined assuming a specific gravity of 1.01. Concentration of each collected sample will be used to determine a cumulative amount excreted as follows:

$Ae_{0\text{-}24,compound} = \sum^{time} Urine_{weight,time} * 1.01 * Conc_{urine,time}$

Cumulative amounts of DMA and ranitidine will be log-transformed. A mixed-effect analysis of variance (ANOVA) approach will be used for comparing arithmetic means (for NDMA) and geometric means (for DMA and ranitidine) looking at the effects of treatment, period, and sequence. For NDMA, testing will be one-sided at a 0.05 significance level, and a significant increase will be concluded if the lower bound of the one-sided 95% interval for the arithmetic mean difference excludes 0. For DMA and ranitidine, testing will be one-sided at a 0.05 significance level, and a significant increase will be concluded if the lower bound of the one-sided 95% interval for the geometric mean ratio excludes 1. The results will be transformed back to the original scale by exponentiation to provide treatment geometric means.

Normality assumption of the ANOVA will be verified using the Shapiro-Wilke test for normality and homogeneity of variances will be verified using Levene's test. If either assumption is not valid, a Wilcoxon signed-rank test will be used for the comparison rather than an ANOVA.

Individual and mean time course plots of NDMA, DMA, and ranitidine excreted over 24-hr will be evaluated for each treatment. All urine PK parameters will be summarized using descriptive statistics (number of subjects, mean, SD, coefficient of variation [CV], median, minimum, and maximum) for NDMA, DMA, and ranitidine. Individual time course plots will use actual sampling times. Treatment mean plots will use planned sampling times.

All calculations and the hypothesis testing will be done using R software.

Total fraction of the ranitidine dose excreted in urine ($Fe_{0\text{-}24,ranitidine}$) will be determined for Treatments A and C based on the cumulative amount of ranitidine excreted in urine over 24-h ($Ae_{0\text{-}24,ranitidine}$) divided by 300 mg. This will be calculated for each individual, summarized using descriptive statistics (number of subjects, mean, SD, CV, median, minimum, and maximum), and visualized graphically using box-and-whisker plots.

Renal clearance of ranitidine will be determined (only for Treatments A and C) by calculating the rate of excretion as the difference between the later and earlier compound level divided by the difference in times points (i.e., $[amount_{t2}\text{-}amount_{t1}]/[t_2\text{-}t_1]$). The log-transform of the renal

excretion will be plotted against the midpoint of each time stop. This will be fit to a linear regression using least-squares fitting and the slope and intercept will be used to determine the overall renal clearance. This will be calculated for each individual and summarized using descriptive statistics (number of subjects, mean, SD, CI, median, minimum, and maximum). All calculations will be done using R software.

7.3.2 Plasma Pharmacokinetics

The following PK parameters will be determined for NDMA, DMA, and ranitidine (only Treatments A and C) for each subject based on all available concentration-time points:

- **AUC from time 0 to the sampling time corresponding to the last quantifiable concentration (C_{last}) (AUC_{0-t})**

  AUC_{0-t} will be calculated according to the linear trapezoidal rule from 0 h to the last quantifiable concentration after drug administration.

- **AUC from time 0 extrapolated to infinity (AUC_{0-inf})**

  AUC_{0-t} will be extrapolated to infinity from the AUC from time 0 to the sampling time corresponding to the last quantifiable concentration plus the last quantifiable concentration divided by K_{el}, as follows:

  $AUC_{0-inf} = AUC_{0-t} + C_{last}/K_{el}$

- **Maximum concentration (observed peak drug concentration) (C_{max})**

  C_{max} will be read directly from the observed concentrations.

- **Time at which C_{max} occurs (T_{max})**

  T_{max} will be read directly from the observed concentrations as the blood sampling time corresponding to C_{max}. The earlier time will be reported in cases where the same C_{max} value is observed for a subject at multiple timepoints

- **Terminal half-life ($t_{1/2}$)**

  The terminal half-life will then be calculated using the elimination rate constant as:

  $t_{1/2}=\ln(2)/K_{el}$

- **K_{el}**

  The terminal elimination rate constant, which will be determined from the terminal slope of the log-concentration curve using linear regression

The PK parameters will be analyzed using noncompartmental methods based on actual sampling times. Serum concentrations below the limits of quantification will be set to zero for the purpose of this analysis (see Section 6). AUC_{0-inf}, $t_{1/2}$, and K_{el} for each analyte and subject will only be

included if the subject has 3 or more concentration values on the terminal portion of the pharmacokinetic curve and with an adjusted coefficient of determination (R^2) greater than 0.80.

$AUC_{0\text{-}inf}$ and C_{max} will be compared with either low nitrite/NDMA meals (comparison between Treatments A and B, respectively) or high nitrite/NDMA meals (comparison between Treatments C and D, respectively) using a paired t-test. Normality assumption will be verified using the Shapiro-Wilke test for normality. If the assumption is not valid, the data will be log-transformed, and then tested for normality. Homogeneity of variances will be verified using Lavene's test. If the data in its original or transformed form does not meet the t-test assumptions, a Wilcoxon signed-rank test will be used for the comparison rather than a paired t-test.

These PK parameters will be summarized using descriptive statistics (number of subjects, mean [arithmetic for NDMA; geometric for DMA and ranitidine], SD, coefficient of variation [CV], median, minimum, and maximum) for NDMA, DMA, and ranitidine.

All parameters will be calculated using R software. Mean and individual concentration-time profiles will be presented in graphs.

7.4 Additional Exploratory Analyses

7.4.1 Physiologically-Based Pharmacokinetic Modeling

Plasma and urine NDMA, DMA, and ranitidine concentration data may be used to conduct physiologically-based pharmacokinetic modeling to better understand absorption, distribution, metabolism, and elimination of these compounds. These analyses will be described in more detail in a separate analysis plan.

7.4.2 Urine and Plasma Metabolomics

Profiling metabolites and other molecular constituents in biofluids may have utility for exploratory research. Portions of collected blood and urine samples from this study may be used for the generation of such profiles (e.g., metabolomics) as hypothesis-generating analyses. Other exploratory analyses may also be performed. Additional details regarding the statistical methods for the exploratory analyses will be described in a separate protocol.

7.4.3 Assessment of Meals

All meals prepared for subjects in this study will utilize foods shown in the literature to have low nitrite/NDMA or high nitrite/NDMA content. However, the actual content of nitrites/NDMA in the different meals will not be known at the time of the study. Two servings of each low nitrite/NDMA meal and each high nitrate/NDMA meal from both a washout and treatment day will be frozen to enable potential analysis of nitrite, NDMA and nitrate content. These results would be summarized as listings (i.e., washout/treatment, high/low, breakfast/lunch/dinner/snack) to document the actual intake of nitrites, NDMA, and nitrates in the different meals.

7.5 Safety Analyses

7.5.1 Adverse Events

All adverse events (AEs) will be coded using the latest version of the Medical Dictionary for Regulatory Activities (MedDRA). The incidence of treatment-emergent adverse event (TEAEs), organized by system organ class and frequency, will be summarized by seriousness, severity, relationship to treatment, and by treatment at onset of the TEAE. A detailed listing of serious AEs and TEAEs leading to withdrawal will also be provided.

7.5.2 Clinical Laboratory Tests

Clinical laboratory results (hematology, serum chemistry, and urinalysis) will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum). Clinical laboratory results will be classified as normal or abnormal, according to the reference ranges of the individual parameter. The number and percentage of subjects with abnormal laboratory results will be provided. No statistical testing will be performed on clinical laboratory data.

7.5.3 Vital Sign Measurements

Vital sign measurements and changes from baseline will be summarized using descriptive statistics (number of subjects, mean, SD, minimum, median, and maximum) by treatment and time point.

7.5.4 Safety 12-lead Electrocardiograms

Abnormal 12-lead ECG findings will be recorded as AEs (not planned for each treatment period) in a data listing.

7.5.5 Physical Examinations

Abnormal physical examination findings will be recorded as AEs (not planned for each treatment period).

7.5.6 Other Safety Data

All concomitant medication usage and medications that changed in daily dose, frequency, or both since the subject provided informed consent will be summarized for each subject.

8. Data Quality Assurance

Completed electronic case report forms (eCRFs) are required for each subject randomly assigned to treatment. Electronic data entry will be accomplished through the ClinSpark® remote electronic data capture system, which allows for on-site data entry and data management. This system provides immediate, direct data transfer to the database, as well as immediate detection of discrepancies, enabling site coordinators to resolve and manage discrepancies in a timely

manner. Each person involved with the study will have an individual identification code and password that allows for record traceability. Thus, the system, and subsequently any investigative reviews, can identify coordinators, investigators, and individuals who have entered or modified records.

Furthermore, the investigator retains full responsibility for the accuracy and authenticity of all data entered into the electronic data capture system. The completed dataset and their associated files are the sole property of the sponsor and should not be made available in any form to third parties, except for appropriate governmental health or regulatory authorities, without written permission of the sponsor.

9. Appendices

9.1 Randomization schedule

This is a 4-period, double-blind, crossover study where approximately 18 subjects will be enrolled to receive either ranitidine or placebo administered with meals from a pre-specified menu with foods that are either low or high in nitrites and NDMA. Subjects will be randomized to one of four treatment sequences (i.e., ABCD, ABDC, BACD, or BADC), where treatment codes are summarized in the table below. The sequence randomization is set so that all subjects will be provided low nitrite/NDMA meals for the first two periods of the study and high nitrite/NDMA meals for the last two periods of the study. The first 16 subjects to get enrolled will be randomized in blocks of 4. The remaining two subjects will be randomly placed in 2 of the 4 treatment sequences. The study plans for 1:1 enrollment of males and females, but the randomization will not account for sex and treatment sequence interactions.

Table 3: Treatment Codes and Treatment Names

Treatment Code	Treatment Name
A	Ranitidine (300 mg) + low nitrite/NDMA meals
B	Placebo + low nitrite/NDMA meals
C	Ranitidine (300 mg) + high nitrite/NDMA meals
D	Placebo + high nitrite/NDMA meals

10. References

Zeng T and Mitch WA. 'Oral intake of ranitidine increases urinary excretion of N-nitrosodimethylamine' Carcinogenesis 2016 Jun; 37(6): 625-34