



Plaque Glycolysis and Regrowth Model

Scope /Principle

The *ex vivo* plaque glycolysis and regrowth model (PGRM) is a technique where plaque is sampled from human subjects, after treatment with agents to determine antiglycolytic and plaque growth activities. The purpose of this technique is to provide a simple and quick method for determining if antimicrobial formulations have a direct effect on the metabolic pathways that plaque microorganisms utilize for the production of toxins which adversely affect gingival health and plaque regrowth. The model focuses on the production of organic acids such as lactic, acetic, butyrate, etc. by plaque bacteria and the ability of bacterial plaque to grow after treatment. This method utilizes *in vivo* sampled dental plaque collected from different quadrants in the human oral cavity before and after treatment with various antimicrobial agents. A trial of this *in vivo* model can be completed in one day for 1 treatment. Crossover design studies are conducted for agents where more comprehensive data is needed. These studies last for several days to weeks depending on the number of treatment legs, number of exposures and controls that are evaluated.

References

1. White, DJ, Cox, ER, Liang N, Macksood, D and Bacca L.(1995) A New Plaque Glycolysis and Regrowth Method (PGRM) for the In Vivo Determination of Antimicrobial Dentifrice/Rinse Efficacy Towards the Inhibition of Plaque Growth and Metabolism-Method Development, Validation and Initial Activity Screens. *J Clin Dent.* 6[Spec Issue]:59-70.
2. D'Agostino, RB. Sr., Massaro, JM and Sullivan, LM. (2003). "Non-inferiority trails: design concepts and issues – the encounters of academic consultants in statistics" *Statist. Med.* 22, 169-186.
3. Gupta, G., Hsu, H., Ng., T. Tiwari, J. and Wang, C. (1999). "Statistical Review Experiences in Equivalence Testing at FDA/CBER" American Statistical Association Proceedings of the Biopharmaceutical Section 220-223.
4. Ebbutt, AF. and Frith, L. (1998). "Practical Issues in Equivalence Trials" *Statist. Med.* 17, 1691-1701.

Inclusion Criteria

- Participants are recruited who are not currently taking antibiotics or using Peridex, Listerine mouthrinse, or other antigingivitis/antibacterial oral care products.
- Participants must have at least 16 natural teeth, not be a chronic smoker, or consume alcohol the evening before the test day.
- Participants should not be currently taking antihistamines or cold/allergy products.
- Participants will refrain from preventive dental care for two weeks prior to and throughout the duration of the study.
- Participants will refrain from eating, drinking, flossing and brushing their teeth after 9:00 p.m. the evening before and the morning of the study dates.

Solution Preparation

Plaque Glycolysis Media
Plaque Regrowth Media
Sucrose Solution

0.03% (w/v) BBL Trypticase Soy Broth in water
4.5%(w/v) BBL Trypticase Soy Broth/Sucrose Solution in water
67% (w/v) Sucrose

1) Day before testing:

Measure, at a minimum, 110 ml water and sterilize.
Prepare TSB stock solution by the addition of 1.5 g TSB to 31 ml of DI water and sterilize.
Label microcentrifuge tubes for glycolysis and regrowth samples.

2) Day of testing:

Prepare Sucrose Solution by adding 6 ml of sterile water to 4 g of sucrose. Vortex till dissolved.
Prepare Plaque Glycolysis Media by adding 0.65 ml of TSB stock solution to 99.35 ml sterile water.
Prepare Plaque Regrowth Media by combining 13 ml sterile TSB stock solution and 1 ml of sucrose solution.

Clinical Protocol, Treatments and Plaque Sampling

Participants need to refrain from eating, drinking, flossing and brushing their teeth after 9:00 p.m. the evening prior to and the morning of the study dates.

On the morning of the study, participants take a baseline plaque sample from the upper dentition at the plaque/gum interface (buccal and lingual surfaces) with a sterile cotton swab, being careful to avoid contact with the oral soft tissue.

Participants will break the swab to a one inch length and place in a marked 15 ml centrifuge tube.

Following the baseline plaque sample, participants use their pre-assigned test product.

Dentifrice

Slurry Swishing, participants swish thoroughly with a 1:2 paste:water toothpaste slurry for 60 seconds and expectorate. Slurry volume should be enough to allow for uniform distribution in the oral cavity. Subsequently, the participants rinse their mouths with 10 ml of water for 10 seconds, expectorate, and begin a timer.

Standard Brushing Method, participants brush only the upper dentition previously sampled for baseline plaque with a pre-measured amount of toothpaste (1 – 2.5g) on a standard brush for 30 seconds. Participants swish with the resulting slurry for an additional 30 seconds followed by a 10 ml water rinse for 10 seconds. Following expectoration of the water rinse, participants begin a timer.

Mouth rinses

Participants swish with 10-15 ml of neat rinse for 10-30 seconds and expectorate. Following expectoration of the mouth rinse, participants begin a timer.

Additional plaque samples are taken at 15 and 45 min post-treatment. These subsequent samples are taken from the dentition of the lower left and lower right quadrants respectively. Swabs are broken to a one inch length and placed in corresponding marked centrifuge tubes.

Participants are not allowed to eat, drink, chew gum, or candy during the 45 min post treatment test period.

The study design is a duplicate crossover with a 48 hr washout period between each treatment.

Sample Analysis

- 1) Prepare a glycolysis reference standard by pipetting 1 ml of Plaque Glycolysis Media into a prelabeled microcentrifuge tube.
- 2) Prepare a regrowth reference standard by pipetting 0.3 ml of Plaque Glycolysis Media and 0.7 ml Plaque Regrowth Media into a prelabeled microcentrifuge tube.
- 3) Prepare regrowth samples by pipetting 0.7 ml of Plaque Regrowth Media into each regrowth microcentrifuge tube.
- 4) To each of the centrifuge tubes containing the participants' swabs, add 1.75 ml of the prepared Plaque Glycolysis Media solution. Vortex the samples for 25 sec. After vortexing, transfer the swab sample solution to a 4.5 ml disposable curette with a disposable pipette.
- 5) Record Optical Density (OD) of the swab samples at 600 nm with a spectrophotometer. Normalize each swab sample to an absorbance reading of 0.200 - 0.210 by diluting with Plaque Glycolysis Media. Record the final OD measurement of each adjusted swab sample.
- 6) Transfer 1 ml of the diluted swab sample to a glycolysis microcentrifuge tube.
- 7) Transfer 0.3 ml of diluted swab sample to a regrowth microcentrifuge tube.
- 8) Once all samples have been diluted and allotted, add 50 μ L of sucrose solution to each glycolysis reference and sample microcentrifuge tubes. Vortex each glycolysis microcentrifuge tube for 15 seconds place in a sample shaker at 37°C for 2 hr.
- 9) Vortex each regrowth microcentrifuge tube for 15 seconds. Transfer each regrowth sample to a 1.5 ml disposable curette. Record initial regrowth absorbance at 600 nm. Transfer each regrowth sample back to its respective microcentrifuge tube and place on shaker at 37°C for 4 hr.
- 10) After 2 hr, using a two point calibrated pH electrode (pH 4 and 7), record pH for reference and each glycolysis sample. After 4 hr, using a pellet grinder, disperse samples for 20-30 sec. Transfer regrowth sample to a 1.5 ml disposable curette and record final regrowth absorbance at 600 nm with a spectrophotometer.

Data Calculations:

Plaque Glycolysis: The baseline pH for each subjects set of samples serves as the individuals reference pH. The change in pH, or delta pH, will determine the degree to which plaque glycolysis has been inhibited. This is determined by subtracting the post-treatment pH from the reference pH. The inhibition of plaque glycolysis over a sampling period measures the activity of the test dentifrice. It can be determined by integration of the area under the delta pH sampling time curve (AUC).

$$AUC_{\text{glycolysis}} = 0.5*15(\text{pH}_{15\text{min}} - \text{pH}_{\text{BL}}) + 30*(\text{pH}_{45\text{min}} - \text{pH}_{\text{BL}}) + 0.5*30(\text{pH}_{15\text{min}} - \text{pH}_{45\text{min}})$$

Plaque Regrowth: The numerical value of the initial/final optical densities (OD) and the corresponding sampling time will be tabulated in the Excel spreadsheet. The optical density is then converted to nephelometric turbidity units (NTU). The NTU is calibrated against standard Formazin stock solutions under the same measurement conditions. Assuming the plaque bacterial cells are well dispersed and grown uniformly, the cell concentrations are directly proportional to NTU. The regrowth ratio, r, is defined as the final concentration divided by the initial concentration. This is calculated from the NTU's before and after incubation.

Similar to plaque glycolysis, the regrowth ratio, r, of the baseline sample after incubation is the reference r. The inhibition of plaque regrowth is measured as delta r, or the post treatment r minus the reference r. The inhibition of plaque regrowth over the sampling period measures the activity of the test dentifrices. It can be determined by integration of the area under the delta r-sampling time curve (AUC).

$$AUC_{\text{regrowth}} = 0.5*15(r_{\text{BL}} - r_{15\text{min}}) + 30*(r_{\text{BL}} - r_{45\text{min}}) + 0.5*30(r_{45\text{min}} - r_{15\text{min}})$$

Statistical analysis:

The statistical approach to analyze PGRM performance data should be one that passes effective anti-gingivitis products and fails ineffective products. The high positive correlation between clinical gingivitis and PGRM results suggests that SnF₂ dentifrice and CPC rinse test products that demonstrate positive PGRM results are likely to provide a clinical gingivitis benefit. The specific pass/fail performance rules for SnF₂ and CPC test products can be established using existing statistical methodologies.

For bioequivalence testing, a test formulation and the reference formulation are compared in a study, and equivalence is declared if the test formulation mean is statistically demonstrated to be within a predetermined percentage of the reference mean. A placebo is generally not included in this research since the response would be predictable at near zero. For example, in pharmaceutical applications if no drug is administered then no drug would be measured in the blood.

For clinical equivalence testing, often a placebo will have a small response relative to the positive control. As such, a placebo will often be included in the research. Equivalence can then be determined by demonstrating that a test formulation mean is within a pre-specified range from the positive control reference formulation. That range is often based on the difference between the reference and placebo means. If the objective of the research is to determine true equivalence then the test is two-sided. If the objective of the research is to determine non-inferiority of the test product to the reference product then the test is one-sided and we call the acceptable range the “non-inferiority margin”. This approach is not uncommon in the healthcare research industry, including the asthma and cardiac therapeutic areas (2,3,4).

A proposed approach for clinical gingivitis monograph testing is to reapply the clinical non-inferiority procedure, directly incorporating the reference and placebo observed means in the test, using what we have termed the Non-inferiority PGRM Test. This approach has multiple requirements in a single PGRM clinical study including either a SnF₂ dentifrice or CPC rinse test product, a positive control reference product and a placebo. Each of these requirements can be assessed using analysis of variance for cross-over study designs followed by treatment contrasts. For the reasons described in the previous section, glycolysis was considered the primary analysis variable and regrowth secondary. Since all of the following criteria must be simultaneously met, no multiple comparison adjustments are required.

Glycolysis Activity Requirements

- 1) The positive control treatment* must exhibit statistically greater mean inhibition than the negative control treatment* using a two-sided, 5% type I error rate
- 2) The test treatment* must exhibit statistically greater mean inhibition than the negative control treatment* using a two-sided, 5% type I error rate.
- 3) The test treatment* inhibition must exhibit statistically greater mean inhibition than the average of the positive and negative control treatment* means using a 95% one-sided confidence interval**.

Regrowth Activity Requirements

- 1) The positive control treatment* must exhibit statistically greater mean inhibition than the negative control treatment* using a two-sided, 5% type I error rate.
- 2) The test treatment* must exhibit statistically greater inhibition than the negative control treatment using a two-sided, 5% type I error rate.

* Test SnF₂ treatments are dentifrices; the positive control treatment is a stabilized SnF₂ 0.454% dentifrice and the negative control treatment is a regular dentifrice. Test CPC treatments are rinses; the positive control treatment is a 0.032% bioavailable CPC rinse, i.e. a 0.045% CPC rinse that is 72% bioavailable, and the negative control treatment is deionized water.

** In practice this criteria is satisfied if the 95% lower confidence bound on $\mu_{\text{test}} - \frac{1}{2}(\mu_{\text{pc}} + \mu_{\text{nc}})$ is greater than zero, where μ_{test} , μ_{pc} , and μ_{nc} are the true means for the test, positive control, and negative control treatments.

The proposed statistical criteria require that a test product exhibit plaque regrowth and glycolytic activity statistically superior to that of a negative control and glycolytic activity substantially more similar to that of the positive control than that of the negative control. Based on the relationship between glycolytic activity and clinical activity displayed in Figure 1, it can be concluded that the test treatment will provide clinical efficacy more similar to that of the positive control than that of the negative control.