Optimizing fluoride retention in the mouth of older adults with distinct salivary flow rates

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STATEMENT OF COMPLIANCE

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the IRB for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. All changes to the consent form will be IRB approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title:	Optimizing fluoride retention in the mouth of older adults with distinct salivary flow rates
Study Description:	This study will assess the effect of an improved fluoride mouth rinse (combined with a calcium prerinse) on enhancing the retention of fluoride in the mouth of older adults with saliva flow ranging from normal to hyposalivation. The study hypothesis is that the calcium prerinse will increase the retention of fluoride in the mouth of older adults, when compared with the fluoride rinse alone.
Objectives:	Primary Objective: Determine the benefit of using a calcium mouth rinse, before a fluoride mouth rinse, to enhance fluoride retention in the mouth of individuals with hyposalivation. Secondary Objectives: To determine the role of hyposalivation on the retention of fluoride in the mouth after a fluoride rinse.
Endpoints:	Primary Endpoint: Fluoride concentration in dental plaque (biofilm) up to 2 h after a mouth rinse Secondary Endpoints: Fluoride concentration in saliva up to 2 h after a mouth rinse; Calcium concentration in dental plaque and saliva up to 2 h after a mouth rinse
Study Population:	Twenty individuals, with 65 years of age or more, from both genders, with good general health, independent, with good oral health, from the Ann Arbor, MI area. At least 10 individuals should have signs of hyposalivation (dry mouth).
Phase:	1
Description of Sites/Facilities Enrolling Participants:	Participants will be recruited among patients from the School of Dentistry, University of Michigan, in Ann Arbor, MI.
Description of Study Intervention:	The study will have 2 interventions (crossover design): a. a sodium fluoride mouth rinse at an over-the-counter concentration of 0.05% (226 ppm of fluoride), used for 1 min; b. a calcium mouth rinse (calcium lactate at a

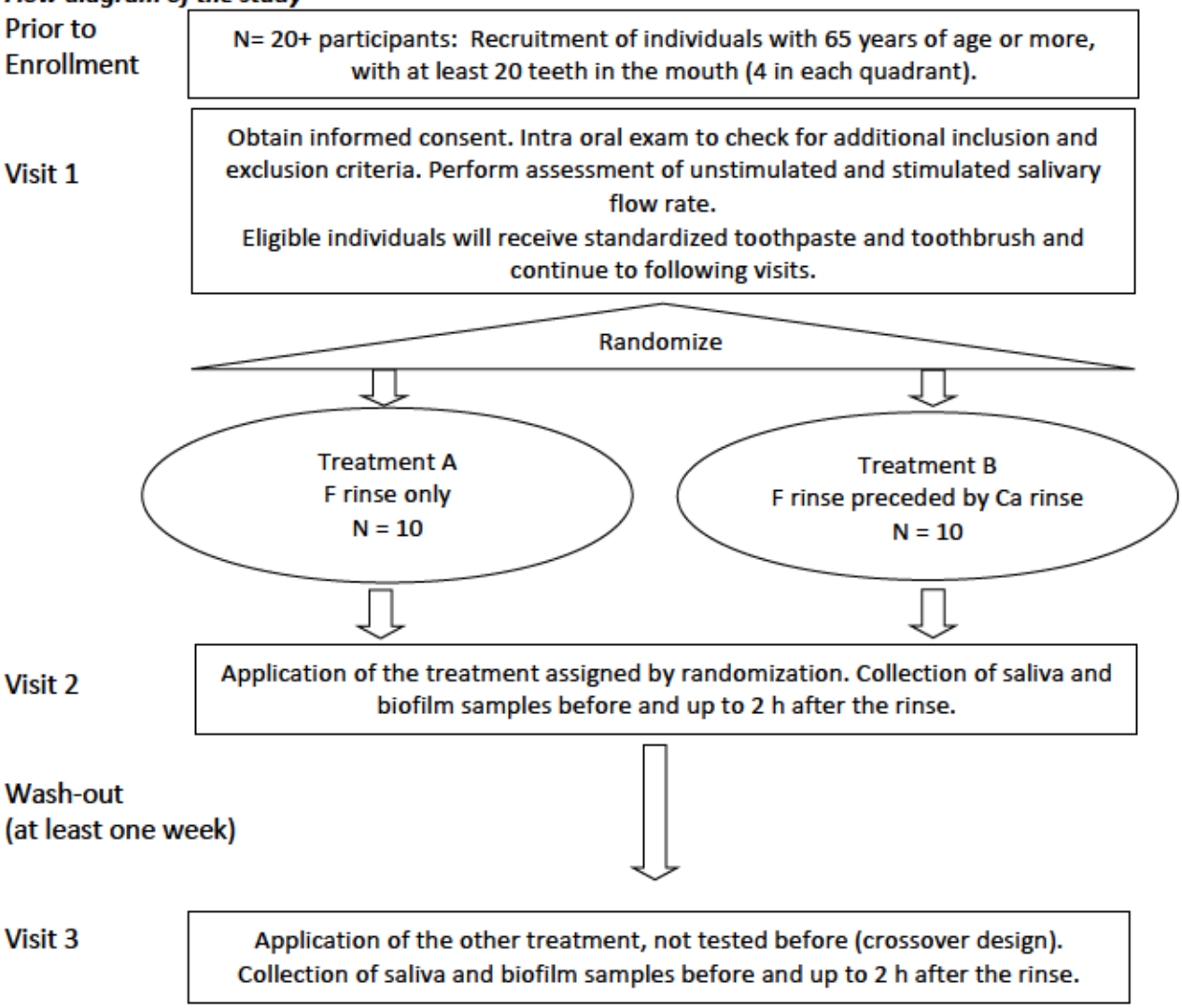
concentration of 150 mM) used for 1 min, followed by the sodium fluoride mouth rinse described in a.

Study Duration: 36 months

Participant Duration: 1 month

1.2 SCHEMA

Flow diagram of the study



1.3 SCHEDULE OF ACTIVITIES (SOA)

Procedures	Baseline, Visit 1 Day 0	Study Visit 2 Day 7 (+1 month)	Study Visit 3 Day 14 (+1 month)
Informed consent	X		
Demographics	X		
Medical history	X		
Intra-oral exam	X		
Saliva flow rate assessment	X		
Randomization	X		
Administer study intervention		X	X
Saliva samples collection		X	X
Dental biofilm samples collection		X	X
Adverse events reporting		X	X

2 INTRODUCTION

2.1 STUDY RATIONALE

Saliva plays a major role in maintaining oral health. A reduction in saliva flow (hyposalivation) drastically increases the risk for oral diseases, including cavities. Hyposalivation (dry mouth) is a special concern for older individuals: about 80% of American people over 65 years of age have at least one chronic condition and 68% have at least two [National Council on Aging, 2015]. Many of the medications used to control these conditions cause hyposalivation [Wolff et al., 2017]. Also, with aging there is a natural exposure of the teeth roots to the oral cavity, and these are more susceptible to cavities due to the higher solubility of their cementum/dentin when compared with the enamel covering the teeth crowns. Therefore, maintaining oral health in older individuals with dry mouth is challenging; the resulting pain, poor nutrition and impaired social functions add to the overall reduction in quality of life in these older adults already suffering from dry mouth.

Fluoride has been successfully used to maintain oral health, especially in dry mouth patients. Yet, the kinetics of fluoride in the mouth of older adults, including those with hyposalivation, is poorly understood. Although they need to use fluoride in higher concentrations, such as prescription toothpastes containing 5,000 ppm of fluoride (F), the extent of the effectiveness of using higher fluoride concentrations in hyposalivation patients has scarcely been explored. Even in individuals with normal salivary flow, the use of a 5,000 ppm F rinse does not result in the expected increased retention of fluoride in the mouth [Staun Larsen et al., 2018], suggesting that there is a limit for increasing intraoral fluoride concentration from oral hygiene products when aiming at increasing their retention. This may be due to the limited supply of positively charged cations, such as calcium, to which fluoride could be bound, to be retained for longer periods in the mouth [Vogel 2011]. Alternative approaches aiming to optimize the fluoride retention by previously supplying a calcium source have been developed [Vogel et al., 2006a, 2006b, 2008a, 2008b], but never tested in hyposalivation patients. For instance, the use of a calcium rinse before a fluoride rinse (Ca→F) successfully enhances fluoride retention in the mouth [Souza et al., 2016], under normal salivary flow conditions. This approach could be particularly helpful for hyposalivation patients, by also providing a supply of calcium ions, which are limited due to the low saliva production.

In the present trial, the effect of salivary flow rate (from normal to hyposalivation) on the retention of fluoride in the mouth of older adults after a fluoride rinse will be studied. We hypothesize that although a reduction in salivary flow reduces the rate of clearance of fluoride from the mouth, the lower availability of calcium ions due to the lack of saliva also reduces fluoride retention. Also, the effect using a calcium rinse before the fluoride rinse to increase fluoride retention in the mouth will be tested in older individuals with a range of salivary flow rates. We hypothesize that the increased retention of fluoride observed after a calcium prerinse will be enhanced in individuals with low saliva flow.

2.2 BACKGROUND

Dry mouth is a serious and prevalent problem in older adults [Furness et al., 2011] and is associated with increased risk for oral diseases, such as caries [Navazesh, 2012]. The lack of saliva reduces the clearance of cariogenic sugars and acids from the dental biofilm, impairs the action of salivary buffering agents and reduces the supply of calcium and phosphate ions, which are natural remineralizing agents [Edgar et al., 2012]. Based on limited evidence, higher fluoride concentration products are usually recommended for older adults with hyposalivation [Zero et al., 2016].

Nevertheless, previous studies have shown that a calcium (Ca) prerinse can boost the retention of F in the mouth [Vogel et al., 2006a; 2006b; 2008a; 2008b; Souza et al., 2016], enhancing its effect. Although the effect of the Ca prerinse on F availability in hyposalivation patients has never been studied, preliminary data give support to the enhanced effect expected for this procedure. Using an in situ model, it was shown that a Ca prerinse used before a F rinse for 14 days increased ~22 and 4-fold the F and Ca concentrations in dental biofilm, respectively [Souza et al., 2016]. This corroborates the hypothesis that the retention of F in the mouth can be enhanced, without the need to increase the F concentration in them. The increased F and Ca retention may be particularly useful for hyposalivation patients.

In the current project we aim to assess the effect of this approach in maintaining increased and sustained levels of Ca and F in the oral fluids of patients with a range of salivary flow rates, varying from normal to hyposalivation. There are no known risks associated with the use of these rinses.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 KNOWN POTENTIAL RISKS

No more than minimal risks are expected to participants enrolled in this trial. The discomforts/risks are listed below:

<u>Immediate discomforts/risks:</u>

1. Discomfort related to refraining from tooth brushing from the previous night until the morning of rinses and sampling collection. This is expected to be a common discomfort (incidence > 25%). The subject may feel malodor in the morning of the test due to the suspension of oral hygiene. After the sampling is completed, the subject will receive a professional cleaning or supervised toothbrushing to remove remnants of dental plaque.
2. Discomfort related to collecting saliva samples. This is expected to be a likely discomfort (10-25%), as some individuals do not feel comfortable in spitting saliva into a tube. This discomfort will be minimized by maintaining the subjected in a private dental chair, with only the trained study staff offering instructions on the procedure.

3. Discomfort related to rinsing with unflavored rinses. This is expected to be a likely discomfort (10-25%), as the rinses will be prepared from reagent grade chemicals, with no flavoring agents.
4. Discomfort related to refraining from eating or drinking for up to 2 hours during the collection of samples. This is expected to be a likely discomfort (10-25%). This may be more disturbing for individuals with reduced saliva flow, if they are used to sip water constantly. If the subject (especially those with dry mouth) feel the need of drink water during the two-hour period after the rinse, he/she will be allowed to do so, and notes taken as protocol deviation in order to later assess their effect on the study outcomes.

Long-range risks:
1. Loss of confidentiality of the study records. This risk is expected to be rare. To minimize the risk of confidentiality breach, data will be stored in password-protected electronic files or locked storage cabinets accessible only to the study personnel. Standards for data collection, management and analysis will be followed.

2.3.2 KNOWN POTENTIAL BENEFITS

The subjects will receive oral hygiene and care instructions provided by the study personnel. Also, in case they have hyposalivation, they will be informed about this, because this condition is not necessarily perceived by the patients and it increases the risk for oral diseases.

The subjects may also benefit from the understanding of the study objectives, making them better aware of the importance taking care of their oral health.

2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

The risks/discomfort associated with this study are no more than minimal. The subjects will be informed about them and should agree to participate before being enrolled. They will benefit from learning about their oral health and good oral hygiene practices.

By investigating an easy and inexpensive procedure to enhance the effect of fluoride, this research will help develop a more effective and cost-benefit way for fluoride use, without the need to increase the fluoride concentration. This potential benefit, especially for vulnerable groups like elders with hyposalivation, outweigh the minimal risks associated with the trial. No risks associated with the use of the mouth rinses are expected.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
Determine the benefit of using a calcium rinse, before a fluoride rinse, to enhance fluoride retention in dental biofilm of individuals with hyposalivation.	Fluoride concentration in dental biofilm (fluid and solid phase) up to 2 h after rinsing with fluoride.	Dental biofilm can take up fluoride and release it for prolonged periods of time, like the hours in between oral hygiene procedures.
Secondary		

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Determine the benefit of using a calcium rinse, before a fluoride rinse, to enhance fluoride retention in saliva of individuals with hyposalivation.	1. Fluoride concentration in saliva up to 2 h after rinsing with fluoride. 2. Calcium concentration in dental biofilm (fluid and solid phase) up to 2 h after rinsing with fluoride. 3. Calcium concentration in saliva up to 2 h after rinsing with fluoride.	1. Salivary fluoride can be considered as a surrogate measure of fluoride availability in the mouth, because from saliva fluoride from rinses gets to dental biofilm. 2. Since a calcium rinse will be used in one of the treatments in this study, determination of calcium concentration in dental biofilm will be made to identify potential sources of fluoride retention (e.g. fluoride bound to calcium ions, calcium fluoride precipitation). 3. Salivary calcium can be considered as a surrogate measure of calcium availability in the mouth; since a calcium rinse will be used in one of the study treatments, salivary calcium will be determined.
Tertiary/Exploratory		
Determine the role of hyposalivation on the retention of fluoride in the mouth after a fluoride rinse	1. Correlation between salivary flow rate and fluoride concentration in dental biofilm up to 2 h after a rinse. 2. Correlation between salivary flow rate and fluoride concentration in saliva up to 2 h after a rinse.	The correlation between salivary flow rate and fluoride in dental biofilm and saliva will be investigated to estimate how the flow rate affect these variables.

4 STUDY DESIGN

4.1 OVERALL DESIGN

The overall hypothesis of this study is that a calcium prerinse used before a fluoride rinse enhances the retention of fluoride in the mouth. Additional hypothesis to be tested is that the reduction in saliva flow (hyposalivation) reduces the clearance and therefore increases the retention of fluoride in the mouth.

The study will be a single-site, phase 1 clinical trial, with a randomized, blinded (regarding the investigators and laboratory analysts) and crossover design. Two intervention groups will be tested: 1. Rinsing with a fluoride mouth rinse only, based on sodium fluoride at an over-the-counter concentration; 2. Rinsing with the same fluoride mouth rinse as in 1, but after a mouth rinse with calcium lactate (a food additive, previously used to enhance the intraoral fluoride retention [Vogel et al., 2006a; 2006b; 2008a; 2008b; Souza et al., 2016]).

For each volunteer, randomization will be used to determine which of the two intervention groups to be tested first. Volunteers randomized to start the trial in group 1, will test group 2 in the following phase, and vice versa. Dental plaque (primary endpoint) and saliva (secondary endpoint) will be collected for fluoride analysis. Calcium concentration in both samples will also be determined (secondary endpoints).

4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

The use of a randomized design is justified to reduce any potential bias in allocating the treatment to the subjects. In addition, by using a crossover design (all subjects exposed to both interventions) it is possible to increase the study power and reduce the variability caused by the volunteer. This is especially important in this trial as people with a range of salivary flow rates (from normal to hyposalivation) will be participating. Since every subject will serve as its own control, the variability due to known (such as salivary flow) and unknown factors can be minimized.

The investigator/analyst will be blind with respect to the allocation of treatments when analyzing the samples for fluoride and calcium concentration. This is to mitigate investigator/analyst bias during the analysis of samples.

4.3 JUSTIFICATION FOR DOSE

Both interventions will be used as mouth rinses, performed for 1 min, which is the typically recommended rinsing time for oral products. The concentrations to be used are also supported by the clinical recommendations and the literature: 1. The fluoride rinse will be used at over-the-counter concentrations of 226 ppm of fluoride (0.05% NaF); 2. The calcium lactate rinse will be tested at 150 mM concentration, which has previously shown the best performance when combined with fluoride in comparison to lower (30 mM) or higher (300 mM) concentrations [Vogel et al., 2006a].

4.4 END OF STUDY DEFINITION

A participant is considered to have completed the study if he or she has completed all phases of the study including the last visit or the last scheduled procedure shown in the Schedule of Activities (SoA), Section 1.3.

The end of the study is defined as completion of the last visit or procedure shown in the SoA in the trial globally.

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Provision of signed and dated informed consent form

2. Stated willingness to comply with all study procedures and availability for the duration of the study
3. Male or female, aged 65 or older
4. In good general health as evidenced by medical history
5. In good oral health as evidenced by a clinical oral exam
6. Having at least 20 teeth in the mouth, being at least 4 (natural or crowned) teeth in all four quadrants of the mouth
7. Having salivary flow rate ranging from normal to hyposalivation according to direct flow rate determination methods
8. Agreement to adhere to Lifestyle Considerations (see below) throughout study duration

5.2 EXCLUSION CRITERIA

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Unable to understand and/or follow study instructions
2. Active periodontitis
3. Oral pain
4. In need of urgent dental care

LIFESTYLE CONSIDERATIONS

During this study, participants are asked to:

- Use a standardized toothpaste and toothbrush, provided by the study team at the first visit, to level the background exposure to fluoride, 7 days prior to each experimental phase
- Refrain from using any other mouth rinse or professional products during the study period
- Refrain from drinking green or black tea (which have high fluoride concentration) on the night before or the day of their visits
- Refrain from brushing their teeth on the night before and the morning of tests/sampling
- Refrain from eating or drinking for 2 hours after the rinses, during which saliva and dental plaque will be sampled

5.3 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention or entered in the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

Individuals who do not meet the criteria for participation in this trial (screen failure) because of active periodontitis, oral pain or need of urgent dental care may be rescreened if they have the exclusion factor treated. Rescreened participants should be assigned the same participant number as for the initial screening.

5.4 STRATEGIES FOR RECRUITMENT AND RETENTION

Participants will be recruited from the clinics of the School of Dentistry, University of Michigan. Primary criteria to be included in the study (having 65 years of age or more, at least 20 teeth, at least 4 teeth in

each quadrant) will be announced to dental providers in the clinics of the School of Dentistry. If identified subjects indicate that they are interested in participating, the provider can refer their name/phone number to the study team for contact and schedule of the first visit. It is expected that a similar number of men and women, and a diverse population in terms of races/ethnicities is recruited.

Participants will be compensated for their participation in the study using a re-chargeable card. They will be compensated with $18.00 for the first visit (checking of inclusion and exclusion criteria), $18.00 for the second visit (test of first intervention group) and $48.00 for the third visit (test of second intervention group). These amounts will be transferred to the re-chargeable card within 2-5 business days of the visit being completed.

6 STUDY INTERVENTION

6.1 STUDY INTERVENTION(S) ADMINISTRATION

6.1.1 STUDY INTERVENTION DESCRIPTION

Fluoride-only rinse: This intervention is a 1-min mouth rinse with 15 mL of a 0.05% sodium fluoride rinse (226 ppm F). This is the same fluoride compound and concentration found in over-the-counter fluoride rinses.

Fluoride rinse following a calcium prerinse: This intervention consists of two 1-min mouth rinses of 15 mL each; the first will be performed with a calcium lactate solution, prepared at the concentration of 150 mM [Vogel et al., 2006a]. Calcium lactate is a food additive and safe for human consumption, although in the current study it will not be ingested, but expectorated (used as a rinse). The second rinse, to be performed within 30 s after the first rinse, will be the sodium fluoride rinse at 226 ppm F, as described above.

6.1.2 DOSING AND ADMINISTRATION

All participants will test both intervention groups; half of them will test the groups in one order (1->2), the other half in the other order (2->1). The order will be determined randomly using a computer-generated randomization list in MS Excel.

Each intervention group will be tested in one experimental phase, spaced at least 7 days from the following phase to avoid any interference with the previous treatment [Fernández et al., 2015].

No treatment will be performed by the patient at home; both interventions will be delivered by the study team at the study visits.

6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

6.2.1 ACQUISITION AND ACCOUNTABILITY

Both rinses to be used in the study will be prepared at the research laboratory of the principal investigator, following guidelines used at the University of Michigan Research Pharmacy. The use of plain mouth rinses (non-commercial, without flavoring agents, surfactants, coloring agents) is required to test the real effect of the chemicals under evaluation (sodium fluoride and calcium lactate), without

interference of ingredients that may affect the salivary flow rate (flavoring agents) or the interaction between calcium and fluoride intended to be tested here (detergents) [Barkvoll et al., 1988].

Calcium lactate, Cat # C8356, Sigma, and sodium fluoride, Cat# 201154, Sigma, will be used for preparing the rinses.

The research laboratory of the principal investigator, located at the University of Michigan School of Dentistry, 1011 N University Ave, room 2310i, 48109-1078, Ann Arbor, MI, will prepare the rinses. An experienced pharmacist from Research Pharmacy visited the laboratory and recommended the preparation of the rinses there. . The source of each prepared and dispensed rinse (drugs lot #) will be noted in a dispensing log. Also, the final use (if used for a mouth rinse, or discarded due to a cancelation in the participant's appointment) will be recorded. Analyses of the calcium and fluoride concentration in each batch of prepared rinses will be made and recorded.

6.2.2 FORMULATION, APPEARANCE, PACKAGING, AND LABELING

The rinses will be prepared to contain 150 mM of calcium lactate and 0.05% of sodium fluoride, respectively.

The 0.05% sodium fluoride rinse will be prepared in one, 1-L batch, to be stored in a plastic flask, with an expiration date of 6 months. From the total volume prepared, 15-mL aliquots to be used by each patient will be dispensed by the PI, according to the patient's schedule.

The 150 mM calcium lactate will be prepared in batches of 100 mL, to be used by patients scheduled in the following 7 days. If not used, the rinses will be discarded. Fifteen mL batches to be used by each participant will be dispensed by PI, according to the patient's schedule.

From preparation until use, dispensed rinses will be stored at the PI's lab, in the refrigerator (calcium lactate rinse) or in a locked cabinet. All flasks will be labeled accordingly.

6.2.3 PRODUCT STORAGE AND STABILITY

A 1-L batch of the sodium fluoride solution will be prepared and stored in a plastic flask at room temperature for 6 months (USP 795). The sodium fluoride solution should be stable for this period of time and no contamination is expected considering the antibacterial action of fluoride [van Loveren, 1990]. The fluoride concentration in this solution will be confirmed using a fluoride electrode, against standards with known fluoride concentration [Nóbrega et al., 2019]. Fifteen mL batches for individual patient use will be dispensed one day prior to the appointment. After 6 months, if the experimental phase is not completed, a new batch will be prepared (amount to be determined by the number of visits to be completed), following the same procedures described above.

For the calcium lactate solution, 100-mL batches will be prepared each week participants are scheduled. This is to ensure that this solution is freshly-used (1-4 days) after preparation. Once prepared, the batches will be stored in the refrigerator, but will be brought to room temperature to be used for mouth rinsing. The calcium concentration in all batches will be checked using the Arsenazo III method for detection of calcium, in a microplate reader, against standards with known calcium concentration [Nóbrega et al., 2019].

6.2.4 PREPARATION

The rinses will be delivered directly to the subjects for mouth rinsing. No other mixing will be performed by the subjects.

6.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

Blinding of the volunteers or clinical staff will not be possible because one of the treatments involves one rinse, the other involves two rinses. Nevertheless, the laboratory analyst will be blind with respect with the samples to be analyzed; for this, the samples will be coded with the participant ID and treatment phase (1st or 2nd, not to be confounded with treatment A or B; the treatment to be used in each phase will be determined randomly for each volunteer).

The order in which the two intervention groups will be tested will be randomized using the MS Excel, using a randomization table containing 3 columns with 20 rows:

1. First column: participant ID number, from 1 to 20
2. Second column: letters A or B (to represent the 2 intervention groups; 10 of each letter)
3. Third column: random numbers created using Excel function "rand"

The third and second column will be selected and sorted by the third column; this will randomize the treatment to be used in the first phase, according to the participant ID. The other intervention group will be tested in the following phase.

6.4 STUDY INTERVENTION COMPLIANCE

There will be no study compliance checks in the study as all procedures will be performed in the clinic, when the subject will be followed by a study team member. Deviations from the protocol during the experimental phases (e.g. intake of water in between sample collection) will be noted in the protocol.

7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

7.1 DISCONTINUATION OF STUDY INTERVENTION

Due to the short-term exposure to the interventions to be tested here (one or two 1-min rinses with the products under test), no discontinuation of the study intervention is expected.

7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request.
An investigator may discontinue or withdraw a participant from the study for the following reasons:

- Significant study intervention non-compliance
- If any clinical unanticipated adverse event (AE) occurs such that continued participation in the study would not be in the best interest of the participant
- Disease progression (oral or general) which requires discontinuation of the study intervention
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation

The reason for participant discontinuation or withdrawal from the study will be recorded on the Discontinuation Case Report Form (CRF). Subjects who sign the informed consent form and are randomized but do not receive the study intervention may be replaced. Subjects who sign the informed

consent form, and are randomized and receive the study intervention, and subsequently withdraw, or are withdrawn or discontinued from the study will not be replaced.

7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for the third study visit (second intervention visit) and is unable to be contacted by the study site staff.

The following actions must be taken if a participant fails to return to the clinic for a required study visit:

- The site will attempt to contact the participant and reschedule the missed visit and counsel the participant on the importance of maintaining the assigned visit schedule and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant (where possible, 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address or local equivalent methods). These contact attempts should be documented in the participant's medical record or study file.
- Should the participant continue to be unreachable, he or she will be considered to have withdrawn from the study with a primary reason of lost to follow-up.

8 STUDY ASSESSMENTS AND PROCEDURES

8.1 EFFICACY ASSESSMENTS

The following assessments and procedures will be conducted during the study:

1. Initial intraoral examination: Recruited subjects will come to a first visit in which the study details will be revised and they will sign the informed consent. Subjects who signed the informed consent will then go through an intraoral examination. The number of teeth in the mouth (minimum of 20) and per quadrant (minimum of 4) will be checked. The presence of active periodontitis or any other tooth-related problem needing urgent care will be used as exclusion criteria. In this case, the participant will be disenrolled from the trial and considered a screen failure.

2. Determination of salivary flow rate: All subjects will have their unstimulated and stimulated salivary flow rates determined in the first visit. This will be done using the draining and the gum-stimulated methods for unstimulated and stimulated salivary flow determination, respectively [Navazesh & Kumar, 2008]. For unstimulated salivary flow, the subjects will sit with the head tilting forward, and all saliva accumulating in their mouth will be passively drooled into a pre-weighted, 15 mL-tube, via a disposable funnel. After 5 min of collection, the volume of saliva will be determined by weight, and the flow rate estimated in mL/min. A value lower than 0.1 mL unstimulated saliva/min will be considered as hyposalivation. For stimulated salivary flow, an inert gum or paraffin base will be used as a masticatory stimulus for saliva production. Subjects will be asked to chew the gum (0.5 g) and spit the saliva produced into 15 mL tubes, for 5 min. A stimulated salivary flow rate of less than 0.5 mL stimulated saliva/min will be considered hyposalivation. Both individuals with normal salivary flow rate and hyposalivation will be enrolled in the study, but at least half of the individuals should have some sign of hyposalivation (see enrollment according to salivary flow rate).

3. Enrollment of participants according to salivary flow rate: A maximum of 10 subjects with normal salivary flow can be enrolled in the study (at least 10 of the enrolled subjects should have hyposalivation). The following table exemplify the screening criteria will be used to consider a subject eligible for the trial according to salivary flow rate:

Table: Example of screening table. Once 10 individuals with normal salivary flow rate are enrolled, the following individuals need to have below normal salivary flow to be eligible.

# screened subject	Salivary flow rate (stimulated and/or unstimulated)	Eligible	Sum of individuals with normal flow rate (should not exceed 10)
1	Normal	Yes	1
2	Below normal	Yes	1
3	Below normal	Yes	1
4	Normal	Yes	2
5	Normal	Yes	3
6	Below normal	Yes	3
7	Normal	Yes	4
8	Normal	Yes	5
9	Normal	Yes	6
10	Below normal	Yes	6
11	Below normal	Yes	6
12	Normal	Yes	7
13	Below normal	Yes	7
14	Normal	Yes	8
15	Normal	Yes	9
16	Normal	Yes	**10**
			Subjects enrolled from this point on must have hyposalivation
17	Below normal	Yes	10
18	Normal	**No**	10
19	Below normal	Yes	10
20	Normal	**No**	10
21	Below normal	Yes	10
22	Below normal	Yes	10
Total number of subjects screened: 22 Total number of subjects enrolled: 20 Number of screen failures: 2			

Considering the population with 65 years of age or more attending the School of Dentistry, it is expected that a low salivary flow is found in more than half of the enrolled subjects (due to the frequent use of medications that cause hyposalivation). Therefore, the recruitment according to salivary flow rate is not expected to result in more than 5 screen failures.

It should be noted that the criterium of having "at least 10 individuals with hyposalivation" enrolled allows for the recruitment of 20 individuals with hyposalivation.

The enrollment table will be checked weekly. If the number of enrolled individuals with normal salivary flow is reaching 10, emphasis on the recruiting of individuals with hyposalivation (as observed by their School of Dentistry providers) will be emphasized.

4. Collection of dental plaque (biofilm) samples: On the 2nd and 3rd visits, participants will have dental plaque samples collected for the analysis of fluoride and calcium in the biofilm fluid and solid phases. The collections will be made at the following timepoints:
    - At the beginning of the session
    - 15 min ± 5 min after rinsing with the intervention group
    - 60 min ± 5 min after rinsing with the intervention group
    - 120 min ± 5 min after rinsing with the intervention group

    The plaque samples will be collected by a licensed professional (dentist). Each sample will be collected from a different quadrant of the mouth. A randomized table will be used to determine the order of collection from each quadrant.
    The participant will be asked to suck and swallow any excessive saliva in his/her mouth and a plastic spatula will be used to scrape the buccal surfaces (facing the cheek) of the teeth in that quadrant of the mouth, collecting any dental plaque accumulated there. This collection is expected to take about 1 min. The pooled plaque sample will be immediately immersed in mineral oil to avoid desiccation. The plaque samples will then be centrifuged to separate the fluid and solid phases. The fluid will be collected using a micropipette, under microscope. Both samples – the fluid and the solids – will be analyzed for calcium and fluoride concentration using a colorimetric method (Arsenazo III reagent) and an ion-selective electrode, respectively.

5. Collection of saliva samples: On the 2nd and 3rd visits, participants will have unstimulated saliva samples collected for the analysis of fluoride and calcium. The collections will start at the following timepoints:
    - At the beginning of the session, immediately after biofilm collection
    - 16 min ± 5 min after rinsing with the intervention group, immediately after biofilm collection
    - 30 min ± 5 min after rinsing with the intervention group
    - 61 min ± 5 min after rinsing with the intervention group, immediately after biofilm collection
    - 90 min ± 5 min after rinsing with the intervention group
    - 121 min ± 5 min after rinsing with the intervention group, immediately after biofilm collection

    For saliva collection, participants will drool any produced saliva into a funnel attached to a tube, for 5 min. The collected sample will be analyzed for fluoride and calcium concentration before and after being centrifuged. An ion-selective electrode and the Arsenazo III reagent will be used in the determination of fluoride and calcium concentrations, respectively.

6. Rinsing with the intervention groups: On the 2nd and 3rd visits, participants will rinse with the assigned mouth rinse (fluoride only or a calcium followed by a fluoride rinse) for 1 min each. The rinses will be performed with 15 mL of the assigned products, as a normal mouth rinse. All rinses will be expectorated. At the end of the 1-min rinsing with the fluoride rinse, a chronograph will be set up to determine the following collections, starting 15 min after the rinse and ending at least 2 h later.

7. Determination of fluoride (F) and calcium (Ca) concentrations in dental biofilm and saliva samples: Biofilm fluid and saliva samples will be assessed for F and Ca concentrations directly. In the solid phase of dental biofilm, F and Ca will be previously extracted from the biofilm with 0.5 M HCl for 3 h at room temperature [Cury et al., 1997; Tenuta et al., 2006] before determination of concentrations in the acid extract. F concentrations will be determined using a F electrode set up for microanalysis [Vogel et al., 1997; Tenuta et al., 2006] using appropriate fluoride standards and TISAB III as the buffer. Ca concentration in the biofilm fluid and saliva samples will be determined using a calcium microelectrode [Vogel et al., 2000; Tenuta et al., 2010]. In the acid extract of the biofilm solid phase, total calcium concentration will be determined using a colorimetric reaction (Arsenazo III), in a microplate reader [Leitão et al., 2018].

No study results will be communicated to the patients, except for their salivary flow rate, which can significantly increase their risk for caries diseases in case it is low.

8.2 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

IRBMed's guidelines on adverse events (AE) and serious adverse events (SAE) reporting will be followed. These guidelines include the definitions of AE/SAE and when to report it.

8.3 UNANTICIPATED PROBLEMS

IRBMed's guidelines on unanticipated problems (UaP) reporting will be followed. These guidelines include the definition of UaP and when to report it.

9 STATISTICAL CONSIDERATIONS

9.1 Statistical Hypotheses

- Primary Efficacy Endpoint:
- Fluoride concentration in dental biofilm
    - Null hypothesis: A fluoride rinse preceded by a calcium rinse is not superior than a fluoride-only rinse in enhancing fluoride concentration in dental biofilm (fluid and solid phases) up to 2 h after mouth rinsing.
    - Alternative hypothesis: A fluoride rinse preceded by a calcium rinse is superior than a fluoride-only rinse in enhancing fluoride concentration in dental biofilm (fluid and solid phases) up to 2 h after mouth rinsing.

- Secondary Efficacy Endpoints:
- Fluoride concentration in saliva
    - Null hypothesis: A fluoride rinse preceded by a calcium rinse is not superior than a fluoride-only rinse in enhancing fluoride concentrations in saliva up to 2 h after mouth rinsing.
    - Alternative hypothesis: A fluoride rinse preceded by a calcium rinse is superior than a fluoride-only rinse in enhancing fluoride concentrations in saliva (fluid and solid phases) up to 2 h after mouth rinsing.
- Calcium concentration in dental biofilm
    - Null hypothesis: A fluoride rinse preceded by a calcium rinse is not superior than a fluoride-only rinse in enhancing calcium concentration in dental biofilm (fluid and solid phases) up to 2 h after mouth rinsing.

  - Alternative hypothesis: A fluoride rinse preceded by a calcium rinse is superior than a fluoride-only rinse in enhancing calcium concentration in dental biofilm (fluid and solid phases) up to 2 h after mouth rinsing.
- Calcium concentration in saliva
  - Null hypothesis: A fluoride rinse preceded by a calcium rinse is not superior than a fluoride-only rinse in enhancing calcium concentration in saliva up to 2 h after mouth rinsing.
  - Alternative hypothesis: A fluoride rinse preceded by a calcium rinse is superior than a fluoride-only rinse in enhancing calcium concentration in saliva up to 2 h after mouth rinsing.

- Tertiary/exploratory Endpoints:
- Correlation between salivary flow rate and fluoride concentration in dental biofilm
  - Null hypothesis: There is no correlation (beta = 0) between salivary flow rate and fluoride concentration in dental biofilm after rinsing with a fluoride mouth rinse.
  - Alternative hypothesis: There is a correlation (beta ≠ 0) between salivary flow rate and fluoride concentration in dental biofilm after rinsing with a fluoride mouth rinse.
- Correlation between salivary flow rate and fluoride concentration in saliva
  - Null hypothesis: There is no correlation (beta = 0) between salivary flow rate and fluoride concentration in saliva after rinsing with a fluoride mouth rinse.
  - Alternative hypothesis: There is a correlation (beta ≠ 0) between salivary flow rate and fluoride concentration in saliva after rinsing with a fluoride mouth rinse.

9.2 SAMPLE SIZE DETERMINATION

Data from Souza et al., 2016, using rinses similar to be used in this study, were used in sample size calculation. In that study, analysis of fluoride concentration in dental biofilm 10 h after using the rinses was available. (Note that the expected differences between the groups up to 2 h after the mouth rinsing are expected to be bigger, as saliva production overtime tends to clear all substances from the mouth).

Considering an 80% power to detect differences at the 5% significance level, an estimated sample size of 15 was determined (Stata/SE 15.1 for Mac). The sample size was increased to 20 subjects considering an attrition of 30%.

9.3 POPULATIONS FOR ANALYSES

For the primary and secondary outcomes, only participants completing the whole study will provide data for the analyses (per-protocol analysis).

For the tertiary outcomes, a modified intention-to-treat analysis will be used; all volunteers completing the fluoride-only mouth rinse treatment will provide data for the correlation analyses, even if they did not complete the other study treatment.

9.4 STATISTICAL ANALYSES

9.4.1 GENERAL APPROACH

Descriptive statistics: Data of fluoride concentration in saliva and dental biofilm tend to have a not-normal distribution. If that is the case in this study, these data will be presented as the geometric mean

(of log transformed data) and confidence interval (calculated from log transformed data). Data of calcium, if fitting normal distribution, will be presented as means and standard deviations. If not fitting normal distribution, the same approach for fluoride will be used.

Inferential tests: The comparisons between groups will be made using analysis of variance, having the volunteers as statistical blocks (source of variation). This is only to reduce known variability from the error calculation. One-tailed tests will be used to test for superiority of one treatment versus the other. The p-value will be set at 5%.
Before the analysis of variance, the normality of errors will be tested. It is expected that fluoride data is not normally distributed, and therefore a log transformation of data will be performed (which usually is adequate to normalize the data). The same approach will be used for the calcium data.

Besides the groups comparisons, the salivary flow rate will be correlated with the fluoride concentration in dental biofilm and saliva, to help understand the effect of flow rate on fluoride salivary clearance. For that, a regression analysis will be used to correlate the data of salivary flow with fluoride concentration (area under the curve of fluoride concentration over time) in saliva and dental biofilm. A significance level of 5% will be used in this analysis.

9.4.2 ANALYSIS OF THE PRIMARY EFFICACY ENDPOINT(S)

Fluoride concentration in dental biofilm (primary efficacy endpoint) will be determined from the samples collected at baseline and 15 min, 60 min and 120 min (± 5min) after rinsing with the assigned mouth rinse(s). The concentration at each individual timepoint will be compared between the two intervention groups. Also, the area under the curve of fluoride concentration in dental biofilm versus time will be calculated. This single measurement of fluoride bioavailability overtime after the mouth rinse(s) will be compared between the two intervention groups.

Each set of data will be checked for normality of errors. If the data is not normal, it will be log-transformed. An analysis of variance will be conducted, considering as factors the participant (experimental block) and the treatment. The use of the participant as a source of variation aims solely to reduce unknown variability in the error. An alpha level of 5% will be used to determine significant differences between the two groups.

In case data were log-transformed to fit the statistical analysis, their geometric means and confidence intervals (calculated from the log-transformed data) will be presented.

If data is missing from one of the intervention groups, the rest of the participant data (from the other intervention) will be excluded from the analysis.

9.4.3 ANALYSIS OF THE SECONDARY ENDPOINT(S)

Fluoride and calcium concentrations in saliva (secondary endpoints) will be determined from the samples collected at baseline and 16 min, 30 min, 61 min, 90 and 121 min (± 5min) after rinsing with the assigned mouth rinse(s). The concentration at each individual timepoint will be compared between the two intervention groups. Also, the area under the curve of fluoride and calcium concentration in saliva versus time will be calculated and compared between the two intervention groups.

Calcium concentration in dental biofilm (primary efficacy endpoint) will be determined from the samples collected at baseline and 15 min, 60 min and 120 min (± 5min) after rinsing with the assigned mouth

rinse(s). The concentration at each individual timepoint will be compared between the two intervention groups. Also, the area under the curve of calcium concentration in dental biofilm versus time will be calculated and compared between the two intervention groups.

Each set of data will be checked for normality of errors. If the data is not normal, it will be log-transformed. An analysis of variance will be conducted, considering as factors the participant (experimental block) and the treatment. The use of the participant as a source of variation aims solely to reduce unknown variability in the error. An alpha level of 5% will be used to determine significant differences between the two groups.

In case data were log-transformed to fit the statistical analysis, their geometric means and confidence intervals (calculated from the log-transformed data) will be presented.

If data is missing from one of the intervention groups, the rest of the participant data (from the other intervention) will be excluded from the analysis.

10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

10.1.1 INFORMED CONSENT PROCESS

An informed consent describing in detail the study intervention, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study intervention.

10.1.1.1 CONSENT PROCEDURES AND DOCUMENTATION

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be Institutional Review Board (IRB)-approved and the participant will be asked to read and review the document. The investigator will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants should have the opportunity to discuss the study with their family or surrogates or think about it prior to agreeing to participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study. Participants must be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice. A copy of the informed consent document will be given to the participants for their records. The informed consent process will be conducted and the form signed, before the participant undergoes any study-specific procedures. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

10.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be

provided by the suspending or terminating party to study participants. If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants, the Institutional Review Board (IRB), and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

10.1.3 CONFIDENTIALITY AND PRIVACY

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor and their interventions. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or sponsor requirements.

10.1.4 KEY ROLES AND STUDY GOVERNANCE

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10.1.5 DATA HANDLING AND RECORD KEEPING

10.1.5.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the clinical trial staff, under the supervision of the principal investigator. The investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Hardcopies of the study visit worksheets will be provided for use as source document worksheets for recording data for each participant enrolled in the study.

10.1.5.2 STUDY RECORDS RETENTION

10.1.6 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the clinical trial protocol. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

It is the responsibility of the principal investigator to use continuous vigilance to identify protocol deviations. All deviations must be addressed in study source documents. Protocol deviations must be sent to the reviewing Institutional Review Board (IRB) per their policies. The principal investigator is responsible for knowing and adhering to the reviewing IRB requirements.

10.1.7 PUBLICATION AND DATA SHARING POLICY

This trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 3 years after the completion of the primary endpoint by contacting Livia M. A. Tenuta.

10.1.8 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial.

10.2 Abbreviations

AE	Adverse Event
Ca	Calcium
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
F	Fluoride
FDA	Food and Drug Administration
GLP	Good Laboratory Practices
IRB	Institutional Review Board
NaF	Sodium fluoride
NCT	National Clinical Trial
PI	Principal Investigator
SAE	Serious Adverse Event
SOA	Schedule of Activities
UaP	Unanticipated Problem
USP	United States Pharmacopeia

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