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Fluoride content in plaque solids and fluid after ingestion of fluoridated milk

Esperanza A. Martínez-Mier¹, Armando E. Soto-Rojas¹, Christine Buckley¹, Andrea Weitz², Alberto Villa², and Domenick T. Zero¹

 ¹⁾Department of Preventive and Community Dentistry, Oral Health Research Institute, Indiana University School of Dentistry, Indianapolis, IN, USA
 ²⁾Department of Public Nutrition, Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile

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Abstract: This study explored differences in dental biofilm solids and fluid fluoride after ingestion of NaF or Na, FPO, in milk or non-fluoridated milk. Eighteen volunteers ingested 1 mg fluoride in 200 mL of milk or 200 mL of non-fluoridated milk. Biofilm samples were collected at baseline, 30, 60, and 240 min and biofilm solids and fluid were micro-analyzed for fluoride. Analysis of variance was performed and the total delivery, retention, and clearance of fluoride to biofilm solids and fluid were estimated as the area under the curve between 0 and 240 min. No statistically significant differences were found for baseline values. Biofilm fluid fluoride values ranged from 0.11 ± 0.05 to $0.21 \pm 0.08 \ \mu g$ F/mL while biofilm solid values ranged from 0.62 ± 0.39 to $1.75 \pm 1.16 \ \mu g \ F/g$. Biofilm fluid values were significantly lower at 60 min for Na₂FPO₃ in milk. Clearance profiles for biofilm fluid diverged after the initial 60 min. Na, FPO, had a smaller area under the curve from 60 to 240 min than NaF. Results of this study indicate that the release and clearance of fluoride in biofilm vary among fluoridation compounds and that the concentrations in biofilm solids and fluid also vary and are not correlated to each other in many cases.

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Fax: +1-317-274-5425 E-mail: esmartin@iu.edu

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Introduction

Fluoride is widely recognized for reducing the prevalence of dental caries. Results of numerous studies have shown that fluoride decreases the incidence of dental caries and slows or reverses the progression of existing lesions by decreasing the rate of enamel demineralization and enhancing the rate of enamel remineralization (1-3). Current understanding of the mechanism of action of fluoride indicates that its major effect is post eruptive and topical, and that this depends on fluoride being present in the biofilm/enamel interface in adequate amounts during caries formation and reversal (1).

There appears to be a general consensus that the levels of fluoride in biofilm, more specifically biofilm fluid, may be directly related to the anticaries effects of fluoride (4). Numerous in vitro studies have shown that the rate of lesion progression and remission can be directly affected by the fluoride content of the fluid phase (5-7). Fluoride delivered to the oral fluids is cleared after a period of time, mainly due to the diluting and washing effect of saliva followed by periodic swallowing, but the rate of this clearance has been shown to be greatly dependent upon the initial concentration of fluoride and vehicle utilized for fluoride administration (8). A study conducted in children by Petersson et al. (9), showed that 2 h after ingestion of 1 mg fluoride from water and milk, fluoride concentrations in saliva and dental biofilm presented a similar increase irrespective of the vehicle. These results and others provided an initial support to the dental caries

Correspondence to Dr. Esperanza Angeles Martínez-Mier, Department of Preventive and Community Dentistry, Oral Health Research Institute, Indiana University School of Dentistry, 415 Lansing Street Indianapolis, IN 46202, USA

preventive effect of fluoridated milk (10).

There have been several studies on the kinetics of fluoride in biofilm fluid after topical fluoride application. Initial studies were conducted by Tatevossian (11), who reported increased fluoride levels in pooled biofilm fluid for 2 h, after rinsing with 399.8 μ g F/mL sodium fluoride (NaF) rinse. Vogel et al. (12) found a strong linear correlation between salivary and biofilm-fluid fluoride after administration of a 0.2% NaF rinse. Direct comparisons have also been made of fluoride in biofilm fluid after sodium monofluorophosphate rinses (13). Further studies have investigated the formation of calcium-fluoride-like deposits after the use of 0.2% NaF rinses (14). Results of these studies and others have reported that a major part of the cariostatic activity of fluoride is a function of its concentration in the fluid environment of the teeth (7).

Vogel et al. (12) demonstrated that concentrations of fluoride in biofilm fluid, biofilm solids and centrifuged saliva varied according to the different sources of fluoride used. They used a calcium fluoride rinse; and a calcium chloride, sodium acetate, sodium hexafluorosilicate, and sodium phosphate rinse. Their results suggested that differences in the release and storage of fluoride derived from the various fluoride compounds could potentially explain differences observed in the different oral fluids. According to Vogel and colleagues' results, a more complete understanding of fluoride's kinetics can be obtained by determining the concentration profiles of fluoride in biofilm fluid as a function of time after the fluoride intake in different vehicles and different fluoridating compounds (12). Therefore, the current study aimed at determining the fluoride concentration in biofilm fluid and solids as a function of time after the ingestion of commonly used fluoridating compounds in milk.

Materials and Methods

Prior to initiation, the study protocol was approved by Indiana University Institutional Review Board (IRB study # 061014). Approval by the Federal Drug Administration (FDA) was requested for the compounding and use for human consumption of fluoridated milk. An Investigational New Drug (IND) exemption was granted by the FDA. As part of a study that investigated fluoridated water and milk, this report presents the results of fluoride content in biofilm after fluoridated milk ingestion. The study was performed at two sites with water fluoridation levels of 1 μ g F/mL and 0.6 μ g F/mL, Indianapolis, USA and Santiago, Chile, respectively. Subjects signed an informed consent and authorization for the release of health information for research form and completed a medical history and inclusion/exclusion procedures. Two days prior to each test day, participants in each site received a professional dental prophylaxis using a non-fluoridated prophylaxis paste followed by a 48 h no-brush period. Upon completion of the dental cleaning subjects were asked to maintain their normal diet but not to drink or eat food higher in fluoride content (for which they were given a detailed list and instructions regarding food high in fluoride content), refrain from brushing their teeth or using any other dental hygiene products for two days before the test day, and to refrain from the use of chewing gum, hard candy or lozenges after 8:00 pm prior to their test day and refrain from eating, smoking or drinking for at least 2 h before their appointment time of the test day. A two week wash out period was left between study legs, where subjects were asked to brush with non-fluoride toothpaste given to them.

Nine subjects per each of the two sites (10 males and 8 females), ages 18 to 55 years old participated in the study. Mean age was 24.1 years old (with a range of 18 to 35 years old); 25.6 years old (with a range of 21 to 35) years old for the Indianapolis subjects and 22.6 years old (with a range of 18 to 29) for the Santiago subjects. On each test day, subjects received an oral soft tissue examination and then, under supervision, ingested 1 mg fluoride in 200 mL (for a concentration of 5 μ g F/mL) of either sodium fluoride (NaF - Orion Thermo Electron Corp, Beverly, MA, USA) in milk, or Na, FPO, (SMFP - Spectrum Chemical MFG. Corp, Gardena, CA, USA) in milk, or non-fluoridated milk as a negative control. Sodium fluoride and SMFP were chosen because they are currently used in milk fluoridation programs. Subjects drank the milk without swirling. Total fluoride analysis by micro-diffusion as described by Taves (15) and later modified by Martinez-Mier et al. (16) were conducted to verify the concentration of compounded solutions during each test visit. Product was dispensed to each subject following a randomization schedule.

A baseline supragingival biofilm sample was collected before dispensing the product. Biofilm collections were then obtained at 30, 60, and 240 min by pooling biofilm from all four quadrants using a pre-determined order. Immediately before dental biofilm collection, the subjects were instructed to swallow all remaining saliva, keep their mouth open and then the collection area was dried or cleaned from saliva using a light stream of air. Buccal and interproximal biofilm samples were collected by a trained dentist. Approximately 1 mg of dental biofilm was collected from the buccal and interproximal surfaces of the anterior and posterior teeth of all four quadrants. Biofilm samples were collected using a standardized approach; collections were performed from the same sites each time. Using a previously constructed plastic strip, pooled biofilm samples were collected from each buccal and interproximal area starting from the upper right quadrant to the upper left, lower left and ending in the lower right quadrants. The pooled biofilm sample was then placed in a sealed pipette tip filled with heavy mineral oil (WSM oil). Samples were carefully stored at -20°C, sealed and in the case of the Santiago site, packaged for transportation in accordance to the International Air Transportation Association (IATA) regulations. Samples were shipped to Indianapolis where analytical procedures were carried out.

A micro analytical method previously described by Vogel et al. (12,17) was used to analyse biofilm samples fluoride content. This method has been reported to have excellent precision and trueness. Prior to sample collection, special centrifuge tubes were constructed by heat sealing 0.01 mL micropipette tips, and were filled with heavy mineral oil. The microcentrifuge tubes containing the plastic strip and biofilm sample were centrifuged for 15 min at 15,000 g at 4°C. Partially oil-filled fine glass micro pipettes were used to recover small aliquots (approximately 0.000005 mL) from the centrifuged tube under a microscope. This constituted the first step of the analytical procedure that allowed a separate assessment of the fluoride-concentration in biofilm fluid. The 0.01 mL micropipette tips were then cut and centrifuged again into a previously prepared centrifuge tube containing mineral oil. A known amount of 1 M HClO₄ was added and mixed by rapid stirring using a 0.25 mL pipette tip and pipettor. Samples were capped and let sit for 1 h. After adding the same volume of a solution 1 M in NaOH and 20% total ionic strength adjusting solution (TISAB III) samples were mixed, centrifuged and the fluid was recovered. This was the second step of the analytical procedure, which provided quantitative information on the size of volume of the fluid reservoir.

Samples were then placed, under mineral oil, on the surface of a specially constructed inverted fluoride electrode. Mineral oil was used to prevent evaporation. TISAB III was added to the samples in a ratio of 9:1. The tip of a micro-reference electrode was placed in contact with the sample to complete the circuit. Triplicate analyses were performed on each pooled biofilm fluid sample. Biofilm and biofilm fluid fluoride was expressed as μ g F/mg or μ g F/mL, which was calculated by comparison to a standard fluoride curve constructed the same day of the analysis.

The reproducibility of triplicate laboratory fluoride analyses in this study was measured using intra-class correlation coefficients (ICC). Two-way analysis of variance (ANOVA) was used to detect the existence of significant differences (P < 0.05) among different fluoride compounds in each of the parameters analyzed when results passed the normality test. The Tukey's test was used for pairwise multiple comparisons to find significant differences (P < 0.05) between each pair of groups. Correlation analysis was done for biofilm and biofilm fluid values at each time point. When the results fail the ANOVA normality test, the Kruskal-Wallis one-way ANOVA on ranks was used to detect significant differences among vehicles, and Dunn's Method was used for pairwise multiple comparisons. The delivery and clearance of fluoride to the oral cavity were estimated as the area under the clearance curve between 0 and 240 min for biofilm fluoride, calculated as the sum of the trapezoidal areas between each pair of sampling time points on the fluoride concentration versus time curve. The delivery and retention parts for the two compounds during the three hour sampling period were different in this study, with the delivery and retention part for NaF and MFP placed at 0-30 min and 0-60 min, respectively. For comparison, two areas were calculated for biofilm solids: Area under the curve (AUC) $_{\rm 0-60\ min}$ and AUC $_{\rm 60-240\ min}.$ The same two areas were calculated for biofilm fluid.

Results

Statistical analyses were performed for the Chile data, the Indianapolis data, and for the two sites combined. No statistically significant differences were found for the Chile and Indianapolis data. Data presented is therefore that of the two sites combined. One volunteer did not complete the study at the USA site. One volunteer at the USA site and two volunteers at the Chile site were disqualified from one leg of the study each, because their baseline values indicated they had not complied with study procedures (tooth brushing was suspected or confirmed prior to biofilm collections). Finally, biofilm fluid from 18 individual samples collections (out of a total of 216 collection points) were not obtained due to problems in transport of the sample or collection. Complete data for biofilm solids samples were obtained for 15 subjects, while complete biofilm fluid data were obtained for 14 subjects.

Triplicate fluoride measurements from each subject were averaged. The ICC for repeated analyses was 0.99. A second technician repeated 10 percent of the samples and the ICC was 0.98. No statistically significant differences were found between study sites and hence the values were combined. The average values of the fluoride concentrations in biofilm solids and fluid at baseline, 30,

Table 1 Mean values (\pm SD) for biofilm solids fluoride (µg F/g biofilm)

Time ^a	NaF-milk	SMFP-milk	Non-fluoridated milk b
Baseline	1.11 ± 0.73	0.73 ± 0.49	0.63 ± 0.42
30 min	1.75 ± 1.16	1.34 ± 0.82	0.94 ± 0.83
60 min	1.53 ± 1.18	1.66 ± 0.97	0.77 ± 0.82
240 min	1.01 ± 0.86	0.97 ± 1.07	0.62 ± 0.39

n = 15 subjects with complete data. Statistically significant by ANOVA ^a baseline < 30 min, baseline < 60 min, 240 min < 60 min and 240 < 30 min (P < 0.0001); ^b Non-fluoridated milk < all other products (P = 0.001)

Table 2 Mean values (± SD) for biofilm fluid fluoride (mgF/mL biofilm fluid)

Time ^a	NaF-milk	SMFP-milk	Non-fluoridated milk ^b
Baseline	0.12 ± 0.06	0.12 ± 0.06	0.11 ± 0.05
30 min	0.19 ± 0.05	0.14 ± 0.05	0.12 ± 0.09
60 min	0.21 ± 0.08	0.13 ± 0.07	0.11 ± 0.09
240 min	0.13 ± 0.06	0.10 ± 0.04	0.12 ± 0.05

n = 14 subjects with complete data. Statistically significant by ANOVA ^a baseline < 30 min, baseline < 60 min, 240 min < 60 min and 240 < 30 min (P < 0.0001); ^b SMFP-milk < NaF-milk at 60 min (P = 0.05); ^c Non-fluoridated milk < all other products (P = 0.001)

60, and 240 min are presented in Tables 1 and 2. No statistically significant differences were found for baseline values among subjects or among collection times for the non-fluoridated milk group for either biofilm solids or fluid.

As expected, the product effect for biofilm solid values was significant (P = 0.0013) indicating that the biofilm fluoride values were lower for non-fluoridated milk at 30, 60, and 240 min when compared to all other products. Although they did not reach significance, the values for SMFP-milk were lower than the values for NaF-milk at 30 and 240 min. The time effect was significant (P< 0.0001) in the following order: baseline values were smaller than 30 and 60 min values. Values at 240 min were smaller than values at 30 and 60 min (Table 1).

The product effect for biofilm fluid was significant for 60 min (P = 0.0468 and P = 0.0336) with SMFP-milk producing lower values than NaF-milk. The time effect was significant for both fluoridated milks (P = 0.0001) in the following order: baseline values were smaller than values at 30 and 60 min. Values at 240 min were smaller than values at 60 and 30 min. For SMFP only, fluoride values at 60 min were smaller than values at 30 min (Table 2).

A statistically significant correlation (r = 0.06, P = 0.03) was observed at 30 and 60 min for the NaF-milk between biofilm fluid and solids values. Values for

 Table 3 Area under the biofilm solids clearance curve for the different compounds and vehicles (untransformed, unadjusted data)

Compound	AUC 0-60 min	SE	AUC 60-240 min	SE
SMFP-milk	76.05	30.26	236.70	79.71
NaF-milk	98.70	43.91	228.60	60.14

n = 15 subjects with complete data. Statistically significant by ANOVA SE = between subject standard error



Fig. 1 Retention and clearance profiles (\pm SD) for biofilm solids fluoride (μ g F/g biofilm).

biofilm fluid or solids were not significantly correlated for any other compounds or time points.

An examination of the clearance profiles for biofilm solids constructed using the logarithm of the concentration of fluoride over time (Fig. 1), showed no evidence of a compound effect on fluoride concentration; with large standard deviations and the different fluoridation compounds overlapping at different time points. An analysis was performed in which the AUC was calculated for 0- to 60-min (delivery and retention phases) and 60to 240-min (clearance phase). Data are presented in Table 3 and Fig. 1. The analysis of variance on these data gave no significant difference for AUC 0-60 min or AUC 60-240 min. An examination of the clearance profiles for biofilm fluid (Table 4 and Fig. 2) showed evidence of a compound effect on fluoride concentration; with the profiles diverging after the initial 60 min (Table 4). The analysis of variance on this data set gave a significant difference for AUC $_{60-240 \text{ min}}$ (P < 0.01) among the different groups, with SMFP having the smallest AUC, indicating slower delivery and clearance (Table 4).

Discussion

Results of the current study indicated that fluoride concentrations varied among different fluoridation compounds at different points in time. The differences observed for the AUC values may indicate a difference in behaviour,

 Table 4
 Area under the biofilm fluid clearance curve for the different compounds and vehicles (untransformed, unadjusted data)

Compound	AUC 0-60 min	SE	AUC 60-240 min a	SE
SMFP-milk	10.35	2.26	31.50	9.31
NaF-milk	10.65	2.80	23.60	9.14

n = 15 subjects with complete data. Statistically significant by ANOVA SE = between subject standard error, ^a SMFP-milk > NaF-milk (P = 0.02)



Fig. 2 Retention and clearance profiles (\pm SD) for biofilm fluid fluoride (μ g F/mL biofilm).

namely delivery and clearance, among compounds. In biofilm fluid after 1 h, fluoride values were smaller for SMFP-milk than for NaF-milk.

Our results are in agreement with those of a publication by Vogel and colleagues (18) who reported that biofilm fluid fluoride concentration is poorly correlated with whole biofilm fluoride content. The authors proposed that the low correlation between total biofilm fluoride and biofilm fluid fluoride suggests that total biofilm fluoride data should be interpreted with caution. The lack of correlation in our results for many time points supports the need to further investigate fluoride concentration in biofilm fluid, specifically.

Our results are in agreement with those of studies that have reported fluoride concentration of NaF as higher than that of SMFP delivered in mouth rinses, as SMFP requires a hydrolysis step to release fluoride to the mouth whereas the NaF does not (13). We hypothesized NaF diffused and more readily interacted with the environment. Based on our results, we propose that the compound used for fluoride delivery in milk may affect its clearance from biofilm.

Vogel et al. (12) found that the average fluoride concentrations in biofilm fluid were significantly different after the calcium chloride, sodium acetate; sodium hexafluorosilicate and sodium phosphate rinse than after sodium fluoride rinse for all the observation times and that the time-response profiles were also different. In the current study, the time response profile for different compounds in milk was also different.

When our biofilm results are compared to those of previous studies, our values are lower to those reported by Vogel and colleagues (12,13,17) and by Petersson et al. (9). However, the studies of Vogel and colleagues relate to a concentrated fluoride rinse and produced very high biofilm fluoride values; therefore, our lower values should not be unexpected. An additional reason for the discrepancies between our study and that of Petersson et al. (9) could be related to the method of analysis. Our samples were micro analyzed utilizing an inverted fluoride electrode, either directly (biofilm fluid) of by acid digestion (biofilm solids). This methodology is similar to that employed by Vogel and colleagues, but the method of choice for the studies conducted by Petersson's group was overnight diffusion. This method could potentially release all extra and intracellular fluoride. Results from an international collaboration have reported that the method of choice plays a significant role in the results of the analysis (16). Our biofilm fluid results could not be compared to those of other studies, since there are no published reports comparing fluid fluoride concentrations after ingestion of fluoridated milk.

Results of this study indicate that the release and clearance of fluoride in biofilm and biofilm fluid seem to vary among different fluoridation compounds and that the concentrations in biofilm and biofilm fluid fluoride also vary and are not correlated to each other in many cases. This study was an initial exploration of fluoride concentrations after fluoridated milk ingestion using different fluoridation compounds. Further studies will be necessary to gain a more thorough understanding of the kinetics of fluoride in biofilm solids and fluid after fluoridated milk ingestion.

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