

Laboratory Procedure Manual

Analyte: **Fluoride, ionic**

Matrix: **Plasma**

Method: **Fluoride Ion-specific Electrode**

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Public Release Data Set Information

This document details the Lab Protocol for testing the item shown in the following table:

| Data File Name | Variable name | Description |
|-----------------------|----------------------|-------------------------------------|
| FLDEP_I | LBDPFL | Fluoride, plasma (umol/L) average 2 |

1 SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

a. *Analyte.*

Fluoride, ionic

b. *Clinical Relevance.*

Fluoride, the ionic form of the element fluorine, is ubiquitous in nature. It is present in all human tissues including plasma. It is not homeostatically controlled so plasma concentrations are determined by the level of current and past intake. Measurement of fluoride in plasma is therefore a biomarker for past and current exposure levels in an individual. One of the major sources of human fluoride exposure is drinking water. There are no known adverse health effects associated with low plasma fluoride levels which typically range from 0.5 to 4.0 $\mu\text{M/L}$. Persistently elevated plasma levels (10 $\mu\text{moles/L}$ or more) for 10 or more years may lead to some degree of skeletal fluorosis.

c. *Assay Principle.*

Fluoride concentrations in plasma and appropriate aqueous standards are measured electrometrically using the ion-specific electrode. Because the limit of detection (LOD) of the electrode, $\sim 1 \mu\text{mole/L}$ (0.019 mg/L), is close to or actually higher than most plasma fluoride concentrations, the hexamethyldisiloxane (HMDS) facilitated diffusion method is employed to quantitatively transfer fluoride from the plasma sample into an alkaline trapping solution of smaller volume. This process results in fluoride concentrations in the solution that is finally analyzed that are well above the LOD and on the linear portion of the standard curve.

2 SAFETY PRECAUTIONS

Safety glasses, gloves and clothing must be worn during the processing of samples by this method.

- a. ***Reagent Toxicity / Carcinogenicity.*** Some of the reagents, namely sodium hydroxide and sulfuric acid, used in this procedure are toxic. Safety precautions must be taken to avoid dermal and inhalation exposure to these reagents. Spills of these reagents must be treated using the acid or base cleanup kits. The reagents used in the analysis of plasma for fluoride are not carcinogenic.
- b. ***Radioactive Hazards.*** This procedure does not use radioactive materials. There are no radioactive hazards associated with it.
- c. ***Biological Hazards.*** This assay involves human plasma samples. Precautions must be followed.

Analysts working directly with the specimens must use proper technique and avoid any direct contact with the sample. Lab coats, gloves and protective eyewear (as required) should be worn while handling the specimens. Plasma spilled on laboratory benches or on any surface must be removed by using a spray of bleach, i.e. 5% Clorox that has been diluted 1:10 with deionized water.

- d. **Chemical Hazards.** MSDSs for HMDS, sodium hydroxide and sulfuric acid are readily accessible on the internet. For example visit the following sites: <http://www.msdssearch.net/MSDSSearch.asp>, or <http://msds.ehs.cornell.edu/msdssrch.asp>. Hardcopies are maintained on file.
- e. **Mechanical hazards.** There are no unusual mechanical hazards associated with this method. Analysts should know and follow the manufacturer's recommendations concerning the safe handling of instruments and other equipment.
- f. **Protective equipment.** Standard safety precautions should be followed when performing this procedure including the use of a lab coat, safety glasses, appropriate gloves, and the use of chemical fume hoods as needed.
- g. **Training.** Training in the use of the multistep HMDS-facilitated diffusion method is required. All analysts must be CLIA-certified and demonstrate proficiency in the analysis before handling samples.
- h. **Disposal of Wastes.** All waste disposals must be in compliance with policies of the Biological and Chemical Safety Committees of Augusta University. Discard waste reagents into an appropriate

container marked for waste handling. Place all disposable items, including vials containing plasma and pipette tips that come in contact with plasma samples, in an appropriate container (red) marked for biological samples. Wipe down all surfaces exposed to plasma with a freshly prepared bleach solution (see item 'c.' above) each day. Non-disposable glassware or other equipment that comes into contact with plasma or other biological samples must be rinsed or wiped with bleach before cleaning and reuse.

3 COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- a. **Software and knowledge requirements.** The initial, handwritten fluoride concentrations of the standards and their associated millivolt values are entered into an Excel spreadsheet. Using the linear regression program, the values for the standards are used to determine the slope and intercept of the standard curve from which the fluoride concentrations of the plasma samples are determined. Knowledge of and experience with the Excel software and its linear regression program and statistical summary program are required for performing these functions.
- b. **Sample information.** All samples are analyzed in runs that include analytical standards, QC bench standards, and QC unknowns (if any). Each plasma sample is identified by a unique code that is generated by NHANES/CDC personnel. Each run is recorded as a file that contains sample ID, analyst's name, volumes, date of analysis and other information. A program generating the database containing this information has been developed by NHANES/CDC/Westat personnel and this fluoride research laboratory.

- c. **Data maintenance.** Following each analytical run, the standards and samples are processed as described above on an Excel spreadsheet, which includes sample file number, sample I.D. and date assayed. The original handwritten mV and other analytical data are maintained in secure files in the fluoride laboratory for at least two years as are copies of the corresponding Excel spreadsheets and the laboratory reports of the plasma concentrations sent to the NHANES/CDC/Westat team.
- d. **Information security.** Information security is provided at multiple levels. Information and analytical findings recorded on paper are maintained in the laboratory which is locked when laboratory personnel are not present. The data accessed via computers require individual login passwords that default to locked conditions during extended periods of nonuse. In addition, the fluoride laboratory is located in the Hamilton Wing of the R&E building, entry into which requires use of a “smart card”. Confidentiality of the results is protected by use of coded ID numbers only. No personal identifiers are ever used.

4 COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

- a. **Special requirements.** There are no special requirements such as fasting or adherence to special diets for this assay. EDTA or heparin is used as the anticoagulant so that plasma rather than serum, which may contain clots, is obtained.
- b. **Sample collection.** The specimen for these analyses is human plasma. Venous blood samples are collected in the standard manner by NHANES personnel in the field. All collection materials with which blood samples come in contact should be pre-screened and approved by this laboratory before they are used to avoid background fluoride contamination issues. Samples should be refrigerated as soon as possible after collection, and frozen for longer term storage. Care should be taken not to completely fill the 5.0-mL tubes to allow for expansion in the freezer.
- c. **Sample handling.** After collection, plasma samples should be frozen at approximately -20°C and shipped with dry ice by overnight air. A packing list showing the code numbers must be included with the samples and the GHSU fluoride research laboratory should be notified before shipment. Unless special arrangements are made, shipment schedules should avoid having samples arrive at the laboratory on weekends or holidays since sample handling at those times may not be possible. After receipt, samples are stored frozen at approximately -20°C until they are prepared for fluoride analysis.
- d. **Sample quantity.** A minimum of 2.0 mL of plasma is needed for duplicate analyses. Vials containing less than 2.0 mL of plasma will be analyzed only once.
- e. **Unacceptable specimens.** Criteria for defining a plasma sample as unacceptable include: (1) use of improper collection materials or techniques leading to elevated fluoride contamination; (2) sample volumes less than the required minimum; or (3) improper shipment or storage of samples leading to thawing for more than one day, leaking or similar problems. All samples are logged in at receipt and problems with shipment or storage are identified at that point. If a sample must be rejected as unacceptable, a description of the problem must be entered into the database and associated with that

sample.

- f. **Long-term stability.** Long-term stability of ionic fluoride in refrigerated or frozen plasma appears to be on the order of several months. The purpose of maintaining plasma samples at low temperatures before analysis is to prevent growth of microorganisms.

5 PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6 PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. *Reagents and Standards. Identification and handling.*

All reagents and standards must be prepared with ACS certified chemicals and deionized water (dH₂O) and stored at room temperature in tightly sealed plastic bottles.

(1) 0.05N NaOH

Using dH₂O, dilute 25.00 mL of 1.00N NaOH to a final volume of 500 mL. Avoid prolonged exposure to air to prevent atmospheric CO₂ from neutralizing the NaOH. Store solution in a closed desiccator containing soda lime.

(2) 0.20N Acetic Acid (CH₃COOH)

To 200 mL of dH₂O, add 5.75 mL of glacial acid. Allow to cool to room temperature. **Adjust final volume to 500 mL using dH₂O.**

(3) Sulfuric Acid (H₂SO₄) Saturated with HMDS

Prepare, handle and store this solution acid in a separately funnel. While preparing or handling the acid, goggles and examination gloves must be worn. Add 300-400 mL of dH₂O to a 1.0 L plastic TriPour beaker containing a spin bar. Place the beaker on a magnetic stirrer and begin stirring the dH₂O. **Slowly** add 84 mL of 36N H₂SO₄. **Caution: The solution will become hot.** Adjust the final volume to 500 mL with dH₂O. Cool in a refrigerator for 2-3 hours. Transfer the solution to a separatory funnel and pour 10-15 mL of HMDS into the funnel. Replace the funnel's ground glass stopper and shake the solution **vigorously** for 2-3 minutes until hundreds of tiny HMDS droplets are dispersed and visible throughout the acid. During this time invert the funnel and open the stopcock slightly to release pressure every 15-30 seconds. Place the separatory funnel on a ring stand in the fume hood. Leave the funnel's stopper ajar overnight to allow any fluoride (as trimethylfluorosilane) to escape from the funnel. This solution will be stable and useable for several weeks.

(4) 3.0N HMDS-Saturated H₂SO₄

The 3.0N HMDS-saturated H₂SO₄ solution must be prepared immediately **prior to each run**. After determining the number of standards and samples to be analyzed in the run, add the appropriate

volume of dH₂O to a plastic TriPour beaker (3 mL for each standard and sample to be analyzed). Open the stopcock of the separatory funnel and add an equal volume of 6.0N HMDS-saturated H₂SO₄ to the dH₂O. The acid-to-water ratio is 1:1 (v/v). Stir well to mix the solution.

(4) Preparation of Stock Standards

Use only ACS Certified 99% pure sodium fluoride and dH₂O. Standards are made in volumetric flasks (dedicated solely for making standards) starting with serial dilutions of a 100.00 mmol/L sodium fluoride stock solution. To make the 100.0 mmol/L standard, add 4.199 g of sodium fluoride powder to 500 mL of dH₂O in a 1.000 L volumetric flask. Mix vigorously for 2-3 minutes. Adjust the volume to 1.000 L with dH₂O. Mix vigorously for 2-3 minutes.

Make a series of fluoride standards as follows:

| Standard, mmol/L | Add _____ mL of _____ mmol/L standard to _____ mL of dH ₂ O |
|------------------|--|
| 10.00 | 100.0 100.0 900.0 |
| 5.00 | 50.0 100.0 950.0 |
| 1.00 | 100.0 10.0 900.0 |
| 0.50 | 50.0 10.0 950.0 |
| 0.10 | 100.0 1.00 900.0 |
| 0.05 | 50.0 1.00 950.0 |
| 0.01 | 100.0 0.10 900.0 |

The equivalent fluoride concentrations of these seven standards, when expressed as mg F/L (ppm) are: 190, 95, 19.0, 9.50, 1.90, 0.950 and 0.190.

(5) Preparation of Analytical Standards for Diffusion

Three sets of four analytical standards are prepared for each analytical run. These standards are used to generate the standard (calibration) curve from which the amounts of fluoride in the plasma samples will be determined. They are 0.25, 0.50, 1.00 and 2.5 nanomoles of fluoride. They are made by placing 25, 50, 100 and 250 microliters, respectively, of the 0.010 mM sodium fluoride stock solution into 12 appropriately labeled diffusion dishes (Falcon 1007). One set of the four standards is read immediately after the three bench quality control standards have been read. The second and third sets of the four standards are read at the middle and at the end of the analytical run each time plasma samples are analyzed.

b. Controls.

1. ***Quality Control Bench Standards.*** One set of the three bench standards, 0.50, 1.00 and 2.50 nanomoles of fluoride, are prepared and read at the beginning of each analytical run. They are made by placing 50, 100 and 250 microliters, respectively, of the 0.010 mM sodium fluoride stock solution into 3 appropriately labeled diffusion dishes. The purpose of the bench standards, whose fluoride

contents are known to the analyst, is to confirm that the analytical procedure is under control as judged by: (1) historical records of millivolt reading for equivalent concentrations of fluoride and (2) the slope, intercept and linear regression r^2 values of the relationship of the mV readings to the nanomoles of fluoride.

2. ***Quality Control Blind Standards.*** The amounts of fluoride in blind quality control standards are not known to the analyst. They are prepared and inserted into an analytical run by the laboratory director at his discretion. When blind quality control standards are used there will be a minimum of one and a maximum of three such standards. The amounts (nanomoles) of fluoride in the blind standards will be within the range of the analytical standards, i.e. from 0.25 to 2.50 nanomoles.

c. ***Major Instrumentation and Other Equipment.***

Potentiometer. OrionStar, model A211 or similar pH/mV meter.

Ion-Specific Electrode. Orion, 9409 fluoride electrode.

Reference Electrode. Accumet miniature calomel reference electrode.

Rotary Shaker. New Brunswick rotary shaker.

Desktop Computer and Printer with appropriate software.

7 CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. ***Cleaning and calibration***

When not in use the tips (sensing ends) of the fluoride and reference electrodes are stored in 0.01 mM NaF and in saturated KCl solutions, respectively. The fluoride and reference electrodes are rinsed with dH₂O and dried with Kimwipes at the beginning of each analytical run and after reading each standard and each sample.

b. ***Calibration Curve***

A calibration curve for this assay is based on the analysis of the set of standards described in section 6.a.5 above. A set of four standards ranging in value from 0.25 to 2.50 nanomoles is analyzed at the beginning, middle and end of each run.

c. ***Verification***

Initial. The initial accuracy of this method was established by analyzing a series of standards prepared as described above. The resulting calibration curves (mV vs ln of the nanomoles of fluoride) were linear with r^2 values >0.98.

Daily. Prior to assaying each run of plasma samples, the mV results from the fluoride bench

standards and first set of analytical standard are reviewed for acceptable accuracy and precision as judged by the slope, intercept and r^2 of the standard curve and by comparison with historical mV readings from previous runs.

8 PROCEDUR OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

8.1 Setting Up Standards and Samples for Diffusion

1. Using a permanent ink marking pen, label the diffusion dish lids with the
2. Using a soldering iron, burn a small hole (ca. 2 mm diameter) through the lid of the diffusion dish near its periphery. The HMDS-saturated H_2SO_4 will be injected through this hole later.
3. Place 3.0 mL of dH_2O in the bottom of the diffusion dish. It will be used to rinse the pipette tips containing standards and samples to ensure complete delivery.
4. Add the standard (see 6(5) above) or sample, in duplicate dishes, to the dH_2O . Record the volumes of plasma that were added to the diffusion dishes. Ideally, the volume of plasma should not be less than 1.0 mL in each dish. Smaller volumes may contain amounts of fluoride that are near or below the lower LOD.
5. Ring the inside periphery of the lid with a thin, unbroken line of Vaseline.
6. To the inside of the lid, place 50 μL of 0.05N NaOH in three drops.
7. Place the lid securely on the bottom of the diffusion dish. Look at the ring of Vaseline to be sure there are no gaps. If a gap(s) is seen, remove the lid and apply Vaseline to that area.
8. Using a refillable syringe, inject 3.0 mL of 3.0N HMDS-saturated H_2SO_4 through the hole in the lid. The transfer (diffusion) of fluoride from the standard or sample to the NaOH trap begins immediately.
9. **Immediately** cover the hole in the lid with Vaseline to prevent the escape of fluoride.
10. Place the diffusion dishes on a tray. Place the tray on a rotary shaker set at approximately 20 rpm.
11. Allow the diffusion process to continue for at least 3 hours (overnight is acceptable).

8.2 Preparation of the NaOH Trap for Analysis

1. Gently remove the lid from the diffusion dish. Invert the lid.
2. Place 20 μL of 0.20N acetic acid near the three drops of NaOH.
3. Gently tip the lid from side to side to combine the acetic acid and NaOH into one drop. This forms an acetate buffer system with a pH of approximately 5.2.
4. Draw the buffered solution into the pipette tip of an adjustable pipette set at 75 μL .
5. Complete filling the pipette tip to 75 μL with dH_2O . This is necessary because during the overnight diffusion process some of the water in the NaOH trap will escape and be transferred to the acidic solution in the bottom of the dish. The amount lost from the NaOH in the individual diffusion dishes is variable and must be restored to a fixed volume for both the standards and the samples.

6. The solution is now ready for analysis.
7. Do not prepare multiple standards or samples in advance. Prepare and analyze each standard or sample one at a time to reduce the risk of significant evaporation.

8.3 Fluoride Analysis: Determine the Millivolt Potential

1. “Warm up” the fluoride and reference electrodes. This is done by placing the electrodes in the non-diffused standard solutions starting with the lowest and then the progressively higher standards. Rinse and dry the electrodes between exposures to the solutions. Repeat this process 3-5 times until the millivolt readings are reproducible and then record the millivolt readings. Then read the bench standards.
2. Place the electrodes in contact with the 75 μL buffered solution. Gently move the electrodes or the dish lid every 10-15 seconds until a stable millivolt reading is obtained and recorded. Stability is usually obtained within 2-5 minutes.
3. Remove the electrodes from the solution, rinse them with dH_2O and dry with Kimwipes.
4. Drop a small piece of pH paper into the solution that was just analyzed and record the pH which should be between 5 and 6. Lower pH values cause the formation of hydrofluoric acid (HF) which is not detected by the electrode. High pH values cause an overestimation of the fluoride concentration because the electrode responds equally well to hydroxyl ions.

8.4 Calculation of the Fluoride Concentrations

1. Open the StatView or Excel spreadsheet for “Diffusion Analysis” and enter the name of the study, the kind of samples being analyzed, the date, the analyst’s initials, etc.
2. Next column: Enter the identification names or code numbers of the samples.
3. Next column: Enter the amounts of fluoride for the standards (nanomoles or nanograms).
4. Next column: Enter the millivolt readings for the standards and samples.
5. Next column: Enter the volumes or weights of the samples.
6. Convert the amounts of fluoride in the standards to their natural logarithms (\ln).
7. Perform a linear regression analysis with millivolts of the standards and samples as X (independent variable) and the \ln of the standards as Y (dependent variable). Select “predicted values.”
8. The output will be the natural logarithms of the amounts of fluoride in the samples.
9. Calculate the amounts of fluoride in the samples by taking the antilogarithms.
10. Divide the amounts of fluoride by the volumes or weights of the samples to obtain the fluoride concentrations of the samples.

9 REPORTABLE RANGE OF RESULTS

9.1 Linearity Limits

The mV responses of the fluoride electrode to ionic fluoride concentrations are linear, on semi-logarithmic plots, from 3 micromoles per liter to 1,000 millimoles per liter.

9.2 Limit of Detection (LOD)

The lower LOD is approximately 0.25 nanomoles when the volume analyzed is 75 μL , ie, a fluoride concentration of 3.3 $\mu\text{moles/L}$ at the electrode. The upper LOD is 75 nanomoles when the volume analyzed is 75 μL , ie, a fluoride concentration of 1,000,000 $\mu\text{mol/L}$ at the electrode. Thus, the upper LOD will not be encountered when analyzing human plasma using the method described in this Procedure Manual.

9.3 Precision

Precision has been estimated by repetitive analysis of plasma samples (duplicates) and triplicate fluoride standards (see section 6.a.5 above) for each analytical run. Precision has been found to be within $\pm 10\%$ for each standard.

9.4 Accuracy

The accuracy of fluoride analysis of plasma using the fluoride electrode in this laboratory was established monthly for more than 10 years by participating in the CDC's Fluoride Proficiency Program. During this period several hundred water samples were analyzed. Except for 22 results from this laboratory all results were within the target range (target concentration $\pm 5\%$). The results that were outside the target range were just barely outside that range. Accuracy is also shown by consistency of mV readings for given standards at the beginning, middle and end of each run.

9.5 Analytical Specificity

This method uses the fluoride-specific electrode. The only other ion to which the electrode responds is the hydroxyl ion. Interference by hydroxyl ions is eliminated by adjusting the pH of the analyzed solution to approximately 5.0. At this pH the hydroxyl ion concentration, 10^{-9} moles/L, is far below the electrode's lower LOD (3×10^{-6} moles/L).

9.6 Carryover

The fluoride electrode is rinsed and cleaned with deionized water and dried (see above) between the analysis of every standard and sample. There has been no evidence of carryover as judged by duplicate analyses of plasma samples and by repeated analyses of analytical standards at the beginning, middle and end of each run.

9.7 Freeze-Thaw and Storage Stability

Plasma samples are received from Westat on dry ice in the frozen state. They are then placed in the freezer to await thawing at room temperature followed immediately by preparation for analysis. There are no cases in which a sample is thawed, frozen again and then thawed again or repeatedly put through freeze-thaw cycles.

10 QUALITY ASSURANCE AND CONTROL

10.1 Quality Assurance

10.1.1 Safety

Augusta University maintains ongoing BioSafety, Radiation Safety and Chemical Safety programs to assure safe working conditions throughout the University campus. The office telephone numbers for these programs are 706-721-2663, 706-721-9826 and 706-721-9643, respectively. They can be called to answer questions or provide assistance as needed. Classes pertaining to each of these programs are offered on a periodic or as-needed basis.

10.1.2 Specimen Handling

Introduction

The goal of specimen handling is to optimize the accurate and reliable measurement of fluoride in water and plasma samples to be analyzed in the 2012-2017 “Analysis of Fluoride in Water and Plasma” project conducted by NHANES-CDC. The HMDS-facilitated diffusion preparatory method used for the analysis of plasma fluoride is described in section 8 of this Manual.

10.1.3 Identification of Specimens

The labeled and coded water and plasma samples are shipped periodically from Westat on ice packs or dry ice to Dr. Whitford’s laboratory for determination of their fluoride concentrations. Personal identifiers (e.g., names of survey participants) are not included on test specimens. NHANES uses a random internal specimen ID number to identify and track individual samples. The Integrated Survey and Information System (ISIS) system at Westat and the National Center for Health Statistics (NCHS) maintain information that links the specimen ID number to the survey participant’s name.

10.1.4 Questions on Analytical Methods for Fluoride

Dr. Whitford and Ms. Danielle Riley address questions concerning the analytical methods. Detailed information concerning the methods can be found in the SOP document for fluoride analysis.

10.1.5 IRB Review

Introduction

Under a congressional mandate (Section 306 of the Public Health Service Act 42 U.S.C.242k) since 1960, NCHS has collected data on the health of the people of the United States through interviews and extensive physical examinations. Seven surveys using health examination procedures have been completed since 1960. As in previous NHANES programs, the survey's primary purpose is to produce descriptive statistics that can be used to measure and monitor the health and nutritional status of the civilian, non-institutional U.S. population. Data collection for this survey involves about 7,000 survey participants per year, of whom about 5,000 per year are expected to be examined.

Human Subjects Review

The NCHS Ethics Review Board (ERB), as defined by the CDC institutional review board (IRB) criteria, has approved collection and laboratory analysis of all human specimens that are part of the current NHANES survey. The IRB of Augusta University has determined that the analysis of water and plasma for fluoride in Dr. Whitford's laboratory is not "human research."

10.2 Quality Control

The fluoride quantitative analytical procedures involve several operations, or steps, each of which is subject to some inaccuracy or imprecision or to the possibility of a mistake. The immediate aim of quality control is to ensure that the analytical values produced are sufficiently reliable for their intended purpose.

A good quality control program monitors the following five parameters:

1. **Clerical Error:** This includes properly documented acknowledgment of transmittal and receipt of specimens (for example, "logging in"), proper labeling of all specimens, correct assignment of laboratory values to the proper subject ID number, and maintenance of proper records for all specimens for future reference.
2. **Techniques:** This includes continued assurance that all personnel performing an assay understand the principles underlying a particular assay and are cognizant of the proper technique for that assay; that all personnel use the same technique for a particular assay; that there is ready access to a current technique manual; and that periodic review is undertaken to ensure use of the most current and reliable techniques.
3. **Reagents and Materials:** This includes confirmation of commercial standards and controls before they reach the bench; proper labeling of reagents, particularly those prepared in the laboratory; ensuring all reagents in use are not outdated; having an adequate supply of current reliable reagents; proper calibration of equipment, such as pipettes; and proper washing of glassware.

4. **Bench Performance:** This includes the use of controls and standards for each assay performed, a technique based on sound statistical principles which allows the technologist performing the assay to detect error outside of previously determined limits before reporting data; documentation of daily bench performance for detection of less obvious error (particularly those which tend to accumulate over time, so-called “drift”); and established procedures to be followed wherever error is found to exceed previously determined limits.
5. **Instrumentation:** This includes periodic preventive maintenance of all instruments in use in the laboratory and documentation that each instrument is maintaining a previously determined level of each performance at each check.

10.2.1 Bench Controls for HMDS-Facilitated Diffusion Analysis of Plasma

For each run of plasma fluoride analysis three bench standards will be analyzed. The fluoride quantities of these standards will be 0.50, 1.00 and 2.50 nanomoles. They will be analyzed at the beginning of each run (i.e. before the analytical standards that are used to establish the standard curve and before analysis of the plasma samples) to ensure that the analytical system is under good control.

10.2.2 Quality Control Records

Quality control results will be evaluated by Dr. Whitford and also sent to Westat and CDC for evaluation for the degree of analytical adequacy and control. Records of all quality control results are maintained for at least 2 years. A QC logbook is maintained that documents out-of-control conditions and remedial actions taken to correct out-of-control conditions.

10.2.3 Proficiency Testing

There are no proficiency testing programs known to this laboratory for the HMDS-facilitated diffusion preparatory method and subsequent determination of fluoride concentrations in plasma using the ion-specific fluoride electrode.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

11.1 Calibration.

System calibration and general readiness is assessed before each analytical run from a review of the mV readings of the bench standards and the first set of analytical standards. This is done by (1) determining the slope of the mV vs. fluoride concentration curve of the bench standards and mV changes for 2-fold and 5-fold changes in fluoride concentrations of the bench standards and (2) by comparison of the mV readings of the bench standards to historical mV readings of the same standards. The mV change for a 2-fold change in concentration should be 17 ± 1 mV; the change for a 5-fold in concentration should be 41 ± 1 mV. When corrective actions are indicated, they are performed and the system is re-evaluated with additional bench standards until acceptable results are obtained

before plasma samples are analyzed.

11.2 Quality Control.

If the results from analysis of three QC bench samples are outside the acceptable limits, ± 3 standard deviations of the historical means, and a reason is identified for the apparent problem, the problem is indicated and the run is scheduled for repeat sample preparation and analysis for samples that have sufficient volume remaining. If the problem is not identified, sample preparation and analysis is suspended until the problem or problems are discovered and corrected. Any questionable sample identified by QC or individual sample evaluation that cannot be confirmed by repeat analysis is not included in the reportable database of results.

12 LIMITATIONS OF METHOD: INTERFERING SUBSTANCES AND CONDITIONS

The H₂SO₄•HMDS-facilitated diffusion method eliminates the possibility of interferences with the accurate analysis of fluoride in standards and plasma by the fluoride electrode. This is because the fluoride in the original plasma sample is quantitatively transferred to the sodium hydroxide trapping solution without the simultaneous transfer of substances, such as high calcium or aluminum concentrations, that could strongly bind with fluoride. The sodium hydroxide trap is buffered with acetic acid to a pH of 5 prior to contact with the fluoride and reference electrodes. This eliminates interference by high concentrations of hydroxyl ions, which are sensed as fluoride by the electrode. A pH of 5 also eliminates interference by hydrogen ions which combine reversibly with fluoride ions to form hydrofluoric acid ($pK_a = 3.4$), a compound not sensed by the electrode.

13 REFERENCE RANGES (NORMAL VALUES)

Because plasma fluoride concentrations are not homeostatically regulated, there are no “normal” concentrations. The concentrations are dependent on the level of acute and chronic fluoride intake. Years of experience, however, have shown that concentrations generally range from 0.2 to 4.0 $\mu\text{moles/L}$.

14 CRITICAL CALL RESULTS (“PANIC VALUES”)

Not applicable for this procedure.

15 SPECIMEN STORAGE AND HANDLING DURING TESTING

Plasma samples are received frozen and stored frozen at approximately -20°C until analysis. After samples are aliquoted for analysis the remainder of plasma, if any, is returned to the freezer at -20°C for later repeat analysis if required. Plasma samples are appropriately discarded if later analysis is not required and the remaining volume is less than 2.0 mL.

16 ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

No alternative method is available. The test system, however, is robust and durable. During the past 40+ years of experience the only problem that delayed analytical progress was breakage of the miniature calomel reference electrode. For that reason, a backup reference electrode, fluoride electrode and potentiometer are always available.

17 TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALS (IF APPLICABLE).

Not applicable at this time.

18 TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN

ACCOUNTABILITY AND TRACKING

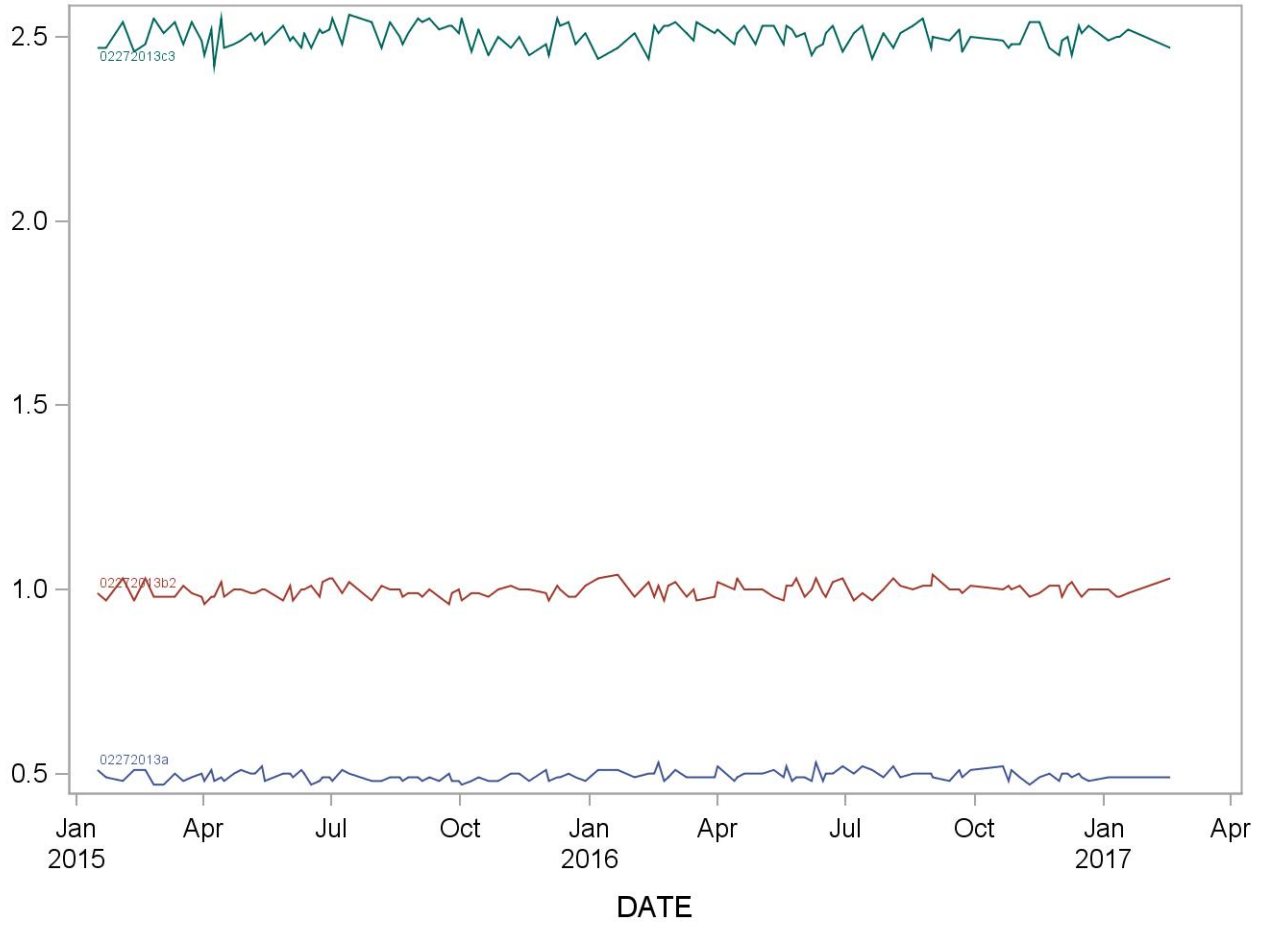
After analysis residual plasma volumes greater than 2.0 mL are held in storage at approximately -20°C in the fluoride laboratory. If the residual volume is less than 2.0 mL the samples are appropriately discarded. Plasma samples are not transferred or referred. Plasma samples are accounted for as described in sections 3 and 4 above.

19 SUMMARY STATISTICS AND QC GRAPHS

See following page.

2015-2016 Summary Statistics and QC Chart for Fluoride (plamsa) umol/L

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|-----|------------|----------|-------|--------------------|--------------------------|
| 02272013c3 | 163 | 16JAN15 | 17FEB17 | 2.501 | 0.032 | 1.3 |
| 02272013a | 163 | 16JAN15 | 17FEB17 | 0.494 | 0.013 | 2.7 |
| 02272013b2 | 163 | 16JAN15 | 17FEB17 | 0.997 | 0.019 | 1.9 |



REFERENCES

Taves DR. Determination of submicromolar concentrations of fluoride in biological samples. *Talanta* 15: 1015-1023, 1968.

Whitford GM. Absorption and plasma concentrations of fluoride. In: *The Metabolism and Toxicity of Fluoride*. Ed HM Myers. Chapter 2, p 10-29. Karger, Basel, 1996.