Determination of Fluorine in Blood Plasma

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A new method for the determination of the total fluorine content of plasma is described. The method involves fixation of the fluorine with low-fluoride calcium phosphate, ashing in a platinum crucible at 500 °C, and the direct determination of the fluoride with the Orion fluoride-ion electrode in a solution of the ashed specimen buffered to pH 5.0. The method is rapid and does not require the isolation of fluoride from other substances. The method has a blank 0.14 μg of fluoride. The relative accuracy of the method is 15.0% and the relative standard deviation is 11.7%.

Although relatively large concentrations of fluorine (20–3000 ppm) are found in biological tissues which calcify, only minute amounts (0.01–0.2 ppm) are present in body fluids and soft tissues (*1*). This circumstance makes it imperative that plasma specimens be carefully protected from contamination by fluorine in the environment if accurate analysis of the native fluoride content is to be reported. Most methods for the determination of total fluorine in plasma have required the incineration of the specimen after the fluoride in the plasma or sera has been "fixed" by combining it with an alkaline substance (CaO, MgO) to prevent the loss of fluoride during the procedure of incineration and the isolation of fluorine from the biological specimen prior to the determination of fluoride.

Many investigators have stressed the reproducibility of their method for the analyses of fluoride in plasma and have compared values obtained by their new method with other methods described in the literature (*2, 3*).

The validity of values for the "fluoride content" of a sample without assurance that the total fluoride or some specific form of fluoride is being determined may not recommend the practical application of a reported method. Extensive fluoride method development for analysis of biological fluids has taken place recently because of the uncertainty as to what form(s) of fluoride are being determined by some generally accepted methods (*4, 5*). These methods have employed a number of unusual approaches to the determination of the fluoride content of plasma and sera.

A simple method of proved reliability that is not tedious and does not require specialized equipment or unusual ability is needed for the determination of total fluorine (ionic plus bound forms) in plasma or sera. Recent studies conducted in our laboratory have established the need for ashing of plasma samples prior to isolation of fluoride (*2, 6*). A variety of techniques have been employed for the isolation of fluoride from the ashed samples including microdistillation (*7*), microdiffusion (*8*), and silanol extraction (*4*). In the simplest conceivable method for the analyses of total fluorine in plasma or sera, the specimen would be ashed with a fixative and the concentration of fluoride in a solution of the ash would be determined with a fluoride-ion specific electrode. Such techniques have not been feasible for the analyses of the fluorine content of plasma because of problems encountered in a measurement of ionic fluoride in a solution of ashed plasma and fixative. The basic technique has been applied successfully to the accurate analyses of fluoride in calcified tissues (*9*) primarily because of the relatively high concentration of fluoride found in hard tissues. The major difficulties with adaption of the techniques used for fluoride analyses of calcified tissue to plasma have been the relatively small concentration of fluoride in plasma, preparation of adequate standards, poor recovery of added fluoride, and failure to obtain reliable repeatable analyses.

This publication will describe a simple technique for the analyses of fluorine in sera which requires the fixation of the fluorine with fluoride–low calcium phosphate prior to ashing. The fluoride is determined with the Orion fluoride ion electrode in a solution of the ashed specimen buffered to pH 5.0. The procedure limits the possibility of fluoride contamination of the specimen, reduces the time required for the analyses of total fluorine in sera, and has proved reliability and accuracy. The method does not require isolation of the fluoride from other substances by diffusion (*8*), distillation (*7*), or an extraction procedure (*4*) prior to the determination of fluorine.

EXPERIMENTAL

Apparatus. The Orion Model 94-09 fluoride-ion electrode and a conventional potassium–chloride reference electrode are employed with an Orion Research Ionanalyzer, Model 801 digital pH meter.

Redistilled Water. Laboratory distilled water is deionized by passing it through a column (36 × 3.4 cm) of filter-ion mixed bed resin (La Motte Chemical Products Co.). The water is then redistilled from an all-glass still, collected, and stored in polyethylene bottles. This water is used in the preparation of all reagents and sample dilutions.

Standard Fluoride Solutions. Dissolve 0.2210 g of reagent grade sodium fluoride in redistilled water and dilute to 1 l. (100 μg F/ml). Dissolve 6.6 g of reagent grade sodium chloride in 500 ml of 0.05 M acetate buffer (pH 5.0). The pH is checked and adjusted to pH 5.0 before final dilution to 1 l. with the buffer. (Final concentrations, 0.11 M sodium chloride and 0.05 M acetate buffer.) Prepare a standard solution in the buffer saline to contain 1.0 μg F/ml. Store all solutions in polyethylene bottles.

Fluoride Low-Calcium Phosphate. Low fluoride-containing calcium phosphate can be prepared by dissolving 89 g of anhydrous $CaCl_2$ in 200 ml of reagent redistilled water, adding 25 g low fluoride containg MgO (0.3–20 ppm F) and boiling the suspension for 30 min. The volume of solution is maintained at the original volume by several additions of water. After heating, the supernatant solution is filtered through 0.45-μ Millipore filter (HAWP 04700) to remove traces of MgO since it is a collector of ionized fluoride. The MgO treatment is repeated to remove as much fluoride from the solution as possible. KH_2PO_4, 72.69 g, is dissolved in 250 ml of water with application of heat, cooled, and the pH adjusted to 7.5 with concentrated NH_4OH (approximately 40 ml). To the phosphate solution add separate volumes of 25 ml of the purified $CaCl_2$ while maintaining the pH at 7.5. Centrifuge aliquots of the calcium phosphate suspension in 250-ml centrifuge tubes for 5 min at 2500 rpm, discard the supernatant, rinse the container, and suspend the precipitate in 150 ml of water, centrifuge, and discard the washing. Repeat this procedure five times and then resuspend the precipitate in about 25 ml of water, transfer each tube to a 600-ml polyethylene beaker, and freeze in a dry ice–ethanol bath so as to distribute the slurry on the sides of the container. Lyophilize for 12 h at 100 μ of mercury and pulverize the cake to approximately 100-mesh size particles in an agate mortar with a pestle.

Solution A. Reagent grade sodium citrate, 5.88 g, $(2H_2O)$, 6.6 g reagent grade sodium chloride, and 500 ml 0.10 N acetic acid are adjusted to pH 5.0 with acetic acid before being diluted to 1 l. (final concentrations, 0.06 N citrate, 0.050 N acetate buffer in 0.16 N sodium chloride).

Solution B. Reagent grade sodium citrate, 5.88 g, $(2H_2O)$ in 500

ml 0.10 N acetic acid is adjusted to pH 5.0 with acetic acid and diluted to 1 l. with redistilled water (final concentration: 0.06 N sodium citrate in 0.05 N acetate buffer).

Solution C. Fluoride low-tricalcium phosphate, 11.4 g, is dissolved in minimal 1 N perchloric acid and diluted to 1 l. with redistilled water.

Materials. Sera were obtained from human volunteers, rats, cows, and rabbits for comparative analytical studies. The human sera were obtained from individuals residing in a fluoridated area for five years. Serum was obtained from each of three steers at slaughter. All other sera were obtained from laboratory animals by heart puncture after ether anaesthesia.

Procedure. The procedure for ashing plasma or serum specimens after fixing the fluoride in the biological specimen with a "fixative" has been previously described (*7*). The procedure has been modified to employ 10 mg of low fluoride-containing $Ca_3(PO_4)_2$ as the fixative for 3–4 ml of plasma of serum. After the sample of blank containing the fixative is ashed, the residue is solutioned with a minimal volume of cold 0.5 N perchloric acid that is added to the platinum dish. All other additions (neutralization, fluoride addition for recovery) are also made in the platinum dish so that the total volume is known. After 4 ml of buffer A solution are added, the pH of the solution is adjusted to pH 5.0, the solution is poured into a 50-ml polyethylene beaker and the fluoride concentration determined with an Orion specific fluoride ion-electrode. The calculation is based upon the total volume of the solution.

Blanks. Blank platinum dishes with 10 mg $Ca_3(PO_4)_2$ are carried through the entire procedure including the fixing and ashing steps. The solids are dissolved in 1 ml of cold (0.5 N) $HClO_4$; 4 ml of Solution A are added, and the pH of the solution is adjusted to 5.0 with 2.5 N NaOH. This usually requires about 170 μl of the base. If fluoride is to be added to the blank after the final pH adjustment, 0.100 ml of 1.0 ppm F solution is added. This amount will permit a satisfactory measurement with the millivolt meter and can be subtracted from the total fluoride found.

Specimens and Recoveries. After the specimens are ashed, add 1.25 ml of cold 0.5 N $HClO_4$, 4 ml of buffer B solution and adjust the solution to pH 5.0 with about 170 μl of 2.5 N NaOH. After the exact amount of alkali required is determined for the first sample, the remainder of the specimens can be adjusted to the proper pH by the addition of the same volume of base.

Specimens in which recovery of fluoride are to be checked should have 0.1 μg F added before the fixation process.

Standard Solutions. These can be prepared directly in the polyethylene beakers. Place 4 ml of buffer A, 1 ml of solution C (11.4 mg $Ca_3(PO_4)_2$/ml), and 0.100 ml of fluoride solution (1 ppm) in the beaker and adjust to pH 5.0 with approximately 170 μl of 2.5 N NaOH. Increments of the fluoride solution are added after each pF reading is obtained. The range of fluoride concentrations used in the standard curve should cover the range expected for samples and recovery specimens. The pF reading for all blanks, standards, specimen, and recovery solutions is recorded after a 30-min equilibrium period.

RESULTS AND DISCUSSION

Although the reactions involved in the fixation of fluoride by calcium phosphate are not known with certainty, the following empirical information is known. Losses of more than 50% of the fluorine of serum occur when no fixative is used prior to ashing (*7*). These earlier observations have been more recently confirmed in this laboratory. The reliability of calcium phosphate as a fixative for sera is supported by the similarity in the results obtained by Venkateswarlu (*5*) for total fluorine analyses of bovine and human sera by both the open ashing procedure employing calcium phosphate as a fixative and an oxygen bomb technique. In the present study 98% of 0.100 μg F added to bovine and rat specimens as NaF prior to fixation was recovered in each of five separate trials and 98 ± 2.3 (SEM)% in seven trials in which 0.200 μg F was added. If phosphoric acid is produced by the reaction of calcium phosphate with the fluorine of the sample, the amount formed (less than 4 μM) would appear to be unimportant. It is probable that any acid produced is buffered by the serum and reacts with available cations in the solid and liquid which were present in the carbonate or bicarbonate forms.

The ionic strength of the standard fluoride solutions and the solutions of ashed sera and fixative and those of the blanks have been adjusted so as to be nearly identical when 3 or 4 ml of sera or plasma are analyzed. The amount of calcium placed in the standard (11.4 mg $Ca_3(PO_4)_2$) compensates for the total amount of calcium and magnesium found in a processed specimen containing the amount of fixative (10.0 mg). The amount of citrate added to the acetate buffer system is slightly in excess of the amount of citrate needed to chelate the cations in solution and therefore does not contribute significantly to a higher ionic strength of the solution and a decrease in activity coefficient of the fluoride ion. Isotonic saline is used as the diluent of the standard to approximate the ionic strength of the specimens and standards. Blanks which are carried through the entire procedure with 10 mg of fixative are diluted with solution A after ashing in order to approximate the monovalent and divalent ionic content of sera. The blank contained on the average 0.14 μg F and proper corrections were made in calculation of the fluorine content of the specimens. Magnesium oxide was tried as the fixative but the results obtained for analyses were variable and recoveries of fluoride added to the specimens were poor.

Table I. Comparison of Fluorine Results Obtained by Different Methods Applied to Bovine Plasma

Method	Description	Mean ± SEM	No. of determns
1[a]	Ashed at 500 °C, F isolated by diffusion, F^- determined colorimetrically.	0.04 ± 0.001	5
2[b]	Ashed at 500 °C, dissolved, and F^- determined with electrode.	0.034 ± 0.0012	11
3	Unashed specimen, F isolated by diffusion, F^- determined with electrode.	0.028 ± 0.0027	4
4	Unashed specimen, F isolated by diffusion, followed by distillation, F^- determined with electrode.	0.035 ± 0.0022	6
5	Unashed specimen, F isolated by diffusion, well contents extracted with silanol, F^- determined with electrode.	0.018 ± 0.0011	4
6	Unashed specimen, F isolated by diffusion, F^- determined colorimetrically.	0.15 ± 0.003	3
7	Ionic F^- determined with electrode.	0.015	

[a] Designated "Reference Method". [b] New method.

The results presented in Table I were obtained by repeated analyses of a large volume of a bovine plasma by the method described here and several generally accepted techniques previously published that employ either specimens ashed with a fixative or unashed specimens from which the fluorine was isolated by diffusion (*8*), distillation (*7*) of pooled diffusates or silanol extraction (*4*) and the fluoride analyzed by colorimetric procedure (*7, 8*) or specific fluoride ion electrode procedures (*10*). In addition, for comparative purposes the ionic fluoride concentration (Method 7, Table I) was determined by direct measurement with the electrode (*10*) in plasma buffered to pH 5.0. This value is obviously less than that found for "total fluorine" with the described technique (Method 2) or Methods 1, 3, and 4. The mean value, the standard error of the mean, and the number of determinations in each replicate study are indicated. Fluoride values obtained with the fluoride electrode can be reported to three places because of the sensitivity of the measurement. The colorimetric procedure is not as sensitive and the concentrations cannot be reported to less than 0.01 ppm. With both techniques, values greater than 0.10 ppm are reported to two significant places. In Method 3, the concentration of fluoride isolated by diffusion techniques was determined with the specific ion electrode rather than the colorimetric procedure described in the publication (*8*)

Table II. Fluorine Content of Plasma of Various Species, ppm

Species	New method	No. of detmns	Ref. method	No. of detmns
Bovine 1	0.039 ± 0.0055	3	0.04 ± 0.003	10
2	0.026 ± 0.0015	3	0.04 ± 0.007	3
3	0.034 ± 0.0012[a]	11	0.05 ± 0.002	6
Rat (pools)				
Low F intake	0.037 ± 0.0053[b]	9	0.05, 0.03	2
High F intake	0.21 ± 0.012	3	0.23, 0.23	2
Rabbit pool				
(High F intake)	0.49 ± 0.024	4	0.52, 0.51	2
Human pool	0.25 ± 0.009	3	0.24 ± 0.020	6

[a] Recovery of 0.100 μg added F^- as NaF. 97.3 ± 0.54% (11) [b] 98 and 99%.

Table III. Comparison of Total Fluorine Determinations in Human Plasma[a] (Single Analysis)

	F^-, ppm		
	Methods		Correlation,
Plasma	Ref.	New	new/ref.
1	0.04	0.059	1.48
2	0.05	0.041	0.82
3	0.09	0.081	0.90
4	0.07	0.070	1.00
5	0.05	0.032	0.64
6	0.07	0.061	0.87
7	0.06	0.048	0.80
8	0.06	0.085	1.42
9	0.08	0.075	0.94
10	0.10	0.12	1.20
11	0.11	0.10	0.91
12	0.07	0.10	1.43
13	0.18	0.14	0.78
14	0.11	0.10	0.91
15	0.11	0.10	0.91
16	0.10	0.12	1.20
17	0.22	0.23	1.05
18	0.10	0.10	1.00
19	0.07	0.10	1.43
Mean (N)	0.090	0.093	1.04
± SEM	± 0.011	± 0.010	± 0.057

[a] Blood collected with EDTA as anticoagulent

(Method 6). An unashed specimen was also analyzed by Method 5. Methods 1 and 4 gave almost identical mean values for the concentration of total fluorine in the specimens utilizing diffusion and microdistillation techniques for fluoride isolation. Since Method 1 is less complicated, it was designated as the Reference for a comparative study with the described method (No. 2). The slightly lower mean value obtained with Method 3 employing an unashed specimen and the electrode has been observed in other comparisons with ashed specimens analyzed similarly. It may be that part of the fluoride evolved by diffusion from unashed samples is in a form that may not be available to the electrode (3). Some support for this suggestion is given by the lower mean value obtained by this method ($p < 0.05$) than by the other methods employed. Method 4 further supports this possibility in that diffusates such as those analyzed in Method 3 were distilled from perchloric acid at 137 °C (7) and gave a slight but significantly higher mean value in the distillate. Method 6, utilizing an unashed specimen, gave a value several fold greater than the other methods. The reasons are not completely known why higher values are obtained for some but not all plasma or sera when an unashed specimen is analyzed. There are indications from our laboratory that some nonfluoride diffusible substance will react in the colorimetric procedure as though it were fluoride.

Several large volumes of heparinized blood from bovine, rats, rabbits, and humans were obtained and the plasma harvested and analyzed repeatedly by the new procedure (Method 2) and the designated Reference procedure (Method 1) in order to test further the proposed method as to its reliability and reproducibility (Table II). It is apparent that the agreement obtained between the mean results of the analyses by the two methods is excellent. The results presented in Table II support the reproducibility and reliability of the new method. The method has a relative accuracy of 15% and a relative standard deviation of 11.7%.

In many fluoride studies with humans it is difficult to obtain sufficient volume of specimen to permit repeated fluoride analyses. Therefore the blood of 19 donors residing in a fluoridated community were collected in a vacuo-tube containing ethylenediaminetetraacetic acid as an anticoagulent and the plasma was harvested. One analysis was made by the Reference method and the described method (Table III) and the correlation between the mean results obtained indicates that a single analysis of plasma or sera by the new method applied to a series of specimen is approximately 104% of the value obtained with the Reference method. The agreement in concentrations of fluoride between the two methods is excellent.

In summary, a reliable method has been developed and applied to the determination of total fluorine in plasma or serum. The method has the advantage of simplicity, eliminates the requirement of isolation of fluoride from other substances, is relatively rapid, and permits a large number of samples to be carried concurrently by one technician.

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