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Measurement of Total and Diffusible Serum Fluoride

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This article describes a technique for the measurement of total and diffusible F content of serum, at clinical significant concentrations of F (1–10 μM). The proposed procedure avoids the interference of unknown serum components with the ion-specific electrode. Sample F is concentrated fivefold through distillation of hydrofluoric acid (Taves' method). Ionic fluoride is presented to the electrode in a simple solution at concentrations within the linear response of the electrode. Average recoveries of F from serum or its ultrafiltrate were 96 ± 7% (21%) and 97 ± 12% (53%) (mean ± SEM [CV]), respectively. With four replicates of each sample, the technique produce within-run standard deviations of 0.6 μM and 2.2 μM at 1 and 10 μM F, respectively. Total precision assessment gave standard deviations of 0.6 μM and 2.6 μM at 1 and 10 μM F, respectively. The fasting serum F levels of normal climacteric women, 45 to 65 years, showed an asymmetric distribution. The data obtained started at the detection limit of the technique (0.1 mM). The 75 percentile was 1.85 μM for total and 0.5 μM for diffusible F. In patients (n = 25) treated with NaF (30 mg F/day) the fasting levels of total serum F (4.5 ± 1.7 μM) did not differ from those of diffusible F (4.2 ± 1.5 μM). In patients (n = 50) treated with sodium monofluorophosphate (15 mg F/day) the fasting levels of total and diffusible serum F were 6.5 ± 1.7 μM and 0.5 ± 0.03 μM, respectively. In conclusion, this paper establishes the presence of two fractions of serum fluorine: diffusible and nondiffusible (or protein bound) and describes a technique for their clinical estimation. In untreated subjects and in patients receiving NaF, the former fraction contains ionic fluoride. In patients treated with MFP, diffusible serum fluorine is composed by ionic fluoride and low molecular weight, peptide-bound, acid-labile fluorine. J. Clin. Lab. Anal. 13:151–157, 1999. © 1999 Wiley-Liss, Inc.

Key words: serum fluoride-protein-bound; osteoporosis; bone mass

INTRODUCTION

Fluoride (F) is used for the treatment of idiopathic and postmenopausal osteoporosis. Sodium fluoride (NaF) or sodium monofluorophosphate (MFP) are the salts more commonly employed. Monitoring serum F of patients has been recommended (1) to avoid toxic levels of the anion.

Serum F is composed by two fractions: diffusible and protein-bound (2–5). Diffusible F has mitogenic activity on osteoblasts (6) and is assumed to be responsible for the increase in bone mass produced by chronic administration of F.

The second fraction, protein-bound F, is assumed to be biologically inactive. For untreated subjects, this fraction has not yet been characterized in terms of their chemical nature, sources, biological significance or fate. Work from this laboratory has shown that this fraction increases in rats and humans treated with MFP (7–9). The hypothesis has been advanced that the levels of nondiffusible F may be clinically important (7–9).

This article reports experiments demonstrating that unknown components of serum interfere in the direct measurement of F with the ion specific electrode. Techniques for the determination of serum diffusible and total F are described and their precision assessed. Values for serum F fractions are reported for untreated and treated (NaF and MFP) climacteric women.

MATERIALS AND METHODS

Influence of the Chemical Composition of F-Containing Solutions on the Accuracy of F Measurements With the Ion-Specific Electrode

NaF solutions were prepared in saline, whole serum or dialyzed serum and distilled water. The latter was used as standard. Two series of F solutions were prepared: 1, 5, 10, and 20 μM and 0.1, 1.0, 5.0, and 10 mM. Ionic strength and pH

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were adjusted by a constant addition (10% v/v) of a concentrated buffer (TISAB) (10). Electrodes were immersed in two ml aliquots of these solutions, following the directions and precautions indicated (10).

A large pool of sera was obtained mixing the remainders of clinical analysis of subjects with normal values of several analytes. Aliquots of this pool were enclosed in cellulose tubing (cut-off MW 12,000) and extensively dialyzed against saline.

Handling of Serum

Blood was drawn with plastic disposable syringes, without anticoagulant. Polypropylene centrifuge tubes (10 ml), Eppendorf tubes, centrifugal filter units, and micropipette tips do not add measurable amounts of F to serum samples.

Ultrafiltration of Serum

The ultrafiltrate of serum was obtained by centrifugation at 1,000*g* for 15 min through Ultrafree-MC centrifugal filter units (Millipore Corp., Bedford, MA) with a 30,000 molecular weight cut-off.

Distillation of F in Biological Fluids

As described by Taves (11), serum or ultrafiltrate aliquots (100 µL) were mixed with 100 µL of 6.0 N hydrochloric acid saturated with hexamethyldisiloxane into distillations chambers (disposable Eppendorf polypropylene tubes, 1.5 mL of capacity, Fig. 1). The F was distilled into the alkali trap (a small polypropylene tube, 100 mL capacity containing 20 mL of 1.65 M NaHO). Distillation was allowed to proceed for six days at room temperature[1]. At the end of this period, distillation was complete. The alkali traps were transferred to a 37°C oven for a day to evaporate the water remaining. A known amount (20 mL) of 2.5 M acetic acid[2] was added to the latter to dissolve the residue. The final solution had a pH of 5.5. Measurement of F was done within 30 min after acetic acid addition. A standard curve was prepared distilling aliquots of NaF standards.

A millivoltimeter with gain setting of ×10 was employed in this work. The latter ensures a minimum reproducibility of ± 0.2 mV, recommended in USP XXII (12) for measurement of F concentrations between 1 and 10 µM. Electrodes were assembled as instructed by Hallsworth et al. (13) to measure 20-mL samples. F was measured with an ion-specific electrode (94-09, Orion Research Inc., Cambridge MA).

[1]Distillation required much longer with Eppendorff tubes time than with the Falcon dishes No. 1007 used by Taves (11). Recovery of 100 nmoles of fluoride after three, four, five, six, and seven days of distillation at 22–24 ºC was 59 ± 19, 88 ± 14, 109 ± 9, 93 ± 12, and 91 ± 5% (mean ± SD, n = 3).

[2]Acetate do not interfere with F measurements (10). The variance of replicates was lower than with the buffer (TISAB) supplied by Orion and the slope agrees with the theoretical value predicted by the Nernst equation (59–60 mV per each order or magnitude in fluoride concentration).

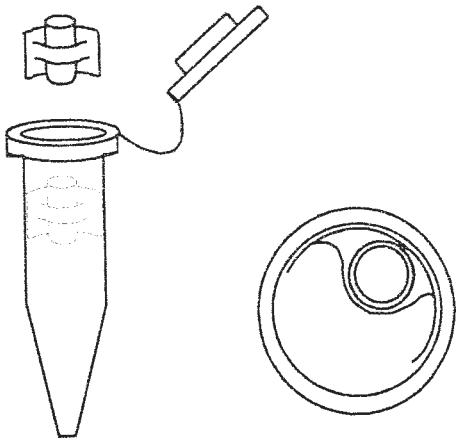

Fig. 1. Distillation chamber. **Left**: an Eppendorf-type polypropilene tube (1.5-ml capacity) containing a small tube (the alkali trap) kept in place by a piece of plastic. **Right**: enlarged view from the top.

Distillation of serum and its ultrafiltrate measures total and diffusible F content. Protein-bound F is determined by the difference of these values.

Recovery of Total and Diffusible Serum F

NaF was added at 1, 2, 4, 6, 8, 10, and 15 mM to serum extensively dialyzed against saline. At each F level, quadruplicate measurements were done of both serum and ultrafiltrates. Average recovery was assessed by the the experimental regression slope between measured vs. theoretical concentration.

Precision of the Measurement of Total Serum F

Two pools of serum extensively dialyzed against saline were made 1 and 10 µM in NaF. One, two, three, and four aliquots at each F level were assayed simultaneously. Within-run and total imprecision were determined as indicated in the NCCLS Tentative Guideline (14).

The procedure proposed requires six days for preparation of sample and distillation, one day for drying of the alkali trap, and one day for the measurement of F. As a rule, each run coincided with the beginning of the next.

Limit of Detection

NaF solutions 10^{-5} M to 10^{-8} M were processed by triplicate as described. The lowest concentration that produced voltage readings significantly different from distilled water (limit of detection with the technique) was 10^{-7} M.

Fasting Levels of Total and Diffusible F in Postmenopausal Women

Samples of sera were obtained from postmenopausal women, 45 to 65 years old, attending the Menopause Clinic

of the University Hospital. We report the values from a series 125 women: 50 controls, 25 treated with sodium F (30 mg F/day), and 50 treated with sodium monofluorophosphate (15 mg F/day) during 6 to 24 months. All these patients had a serum creatinine (15) lower than l.3 mg/dL. The patients were the subjects of a project approved by the Ethics Committe of the Medical School of Rosario.

Statistical Methods

Regression and covariance analysis were done with standard methods (16). Total and within-run imprecision was estimated as indicated in NCCLS Tentative Guideline EP5-T (14).

RESULTS

Unsuitability of the F Electrode for the Direct Measurement of Serum F

In spite of the similarities in F concentration, pH and ionic strength, direct measurement of F showed significant differences in electrode potential between distilled water, saline or whole serum. With whole serum, the slope (Fig. 2, left panel; Table 1) and the apparent F content (Y-intercept) are increased. With saline as solvent, the slope (that measured the sensitivity of the electrode) was reduced to one-fourth. Serum extensively dialyzed against saline gave the same response as saline. Comparison of the latter two curves with that obtained with serum, suggests that unknown serum diffusible species strongly overestimate F concentration. At F concentrations greater than 100 μM, these effects are much less evident (Fig. 2, right panel).

The data displayed in Table 1 convinced us that the precision and accuracy of the F electrode in simple matrix solutions cannot be extended to serum, especially at clinically significant F levels (1–10 μM).

Recovery of Added F

The F content of dialyzed serum with added NaF and their ultrafiltrates (Fig. 3) produced regressions with slopes not significantly different from unity. Average recovery was 96 ± 7% (14%) and 97 ± 12% (15%) (mean ± SEM (CV)) for total and diffusible F, respectively.

Total and Within-Run Precision of the Technique Proposed

The technique described here, doing four replicates of each sample, produced within-run standard deviations of 0.6 μM and 2.2 μM at 1 and 10 μM F, respectively. Total precision gave an standard deviation of 0.6 μM and 2.6 μM at 1 and 10 μM F, respectively (Table 2).

The detection limit depends upon the ratio: sample volume to alkali trap volume. Under the stated laboratory conditions the detection limit is 0.1 μM.

Fasting Levels of Serum (Total and Diffusible) F

Normal fasting levels of total and diffusible F were obtained with residents in the city of Rosario (Argentina). Tap water contains 13 ± 5 μM F. The fasting serum F levels of normal climacteric women, 45 to 65 years, showed an asymmetric distribution. The data obtained started at the lowest detection limit of the technique (0.1 μM). The 75 percentile for untreated postmenopausal women is 1.85 μM for total and 0.5 μM for diffusible F (Fig. 4).

In a group of 25 patients treated chronically with NaF (30

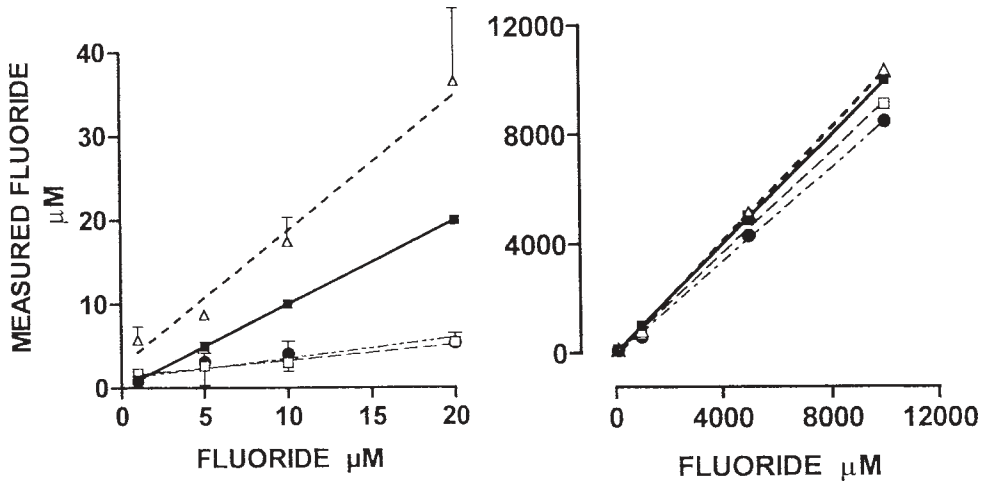

Fig. 2. Standard curves of fluoride. **Left panel**: NaF was dissolved in distilled water (■), human serum (△), saline (□), and human serum extensively dialyzed against saline (●). The apparent fluoride concentrations (vertical axis) in the three latter solutions was determined using the NaF solution in distilled water as standard. **Right panel**: NaF solutions (100—10,000 μM). Same solvents and symbols as in the left panel. In all these experiments ionic strength adjustment was done by addition of one tenth of sample volume of TISAB (10). Bars indicate the standard error of the mean.

TABLE 1. Analytical Data of Regressions: Added vs. Measured F

	Y-intercept ± SE	Slope ± SE	r, *P*
F concentrations: 0–20 μM			
Distilled water	0.08 ± 0.07	0.998 ± 0.015	0.997, < 0.0001
Serum, whole	2.68 ± 2.49	1.608 ± 0.243	0.902, < 0.0001
Serum, dialyzed	1.49 ± 0.70	0.185 ± 0.068	0.651, = 0.022
Saline	1.19 ± 0.84	0.241 ± 0.083	0.675, = 0.016
F concentrations: 100–10,000 μM			
Distilled water	0.07 ± 0.07	0.996 ± 0.023	0.997, < 0.0001
Serum, whole	–67.41 ± 51.00	1.009 ± 0.009	0.999, < 0.0001
Serum, dialyzed	–109.79 ± 86.70	0.920 ± 0.015	0.999, < 0.0001
Saline	–118.19 ± 77.51	0.858 ± 0.013	0.999, < 0.0001

mg F/day), the fasting levels of total serum F (4.5 ± 1.7 μM) were not significantly different from the diffusible F (4.2 ± 1.5 μM) in the same samples.

In another group of 50 patients treated chronically with sodium monofluorophosphate (15 mg F/day), the fasting levels of total and diffusible serum F were 6.5 ± 1.7 μM and 0.5 ± 0.03 μM, respectively.

DISCUSSION

The Specificity of the F Ion Specific Eectrode With Human Serum as Solvent

The F electrode requires adjustment of solutions to pH 5.5. At this pH, F is still ionic and the concentration of hydroxyl ions (to which the electrode is also sensitive) is negligible. As shown in Results, even at pH 5.5, serum contains some unknown diffusible species that interfere with the direct measurement of F. This finding emphasizes the fact that the ion-specific electrode should be used only with very simple solutions, with identical composition in both unknowns and standards. We have found that bactericidal concentrations of sodium azide (anion N_3^-), at pH 5.5, strongly interfered with F measurements. When azide (1.5 or 7.5 mM) was added to 100 μM NaF in distilled water, F values of 72 and 65 μM, respectively, were obtained.

These observations convinced us that the accuracy and precision of the F electrode for the measurement of the anion in very simple solutions, cannot be extended to serum especially at clinically significant F levels (1–10 μM).

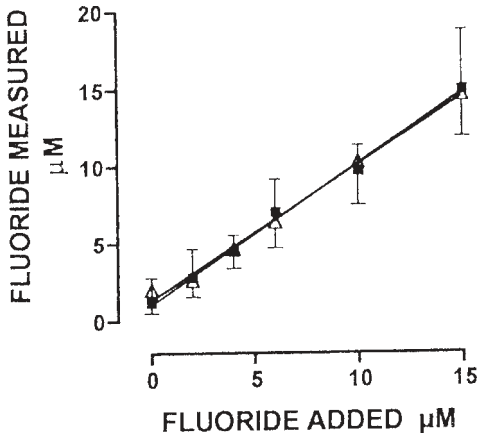

Fig. 3. Recovery of F from dialyzed serum (■) and their ultrafiltrates (△). In these experiments, serum extensively dialyzed against saline was loaded with known amounts of NaF.

F Species in Serum

As stated in the Introduction, there are two fractions of F in human serum (2,3,17,18). One fraction is diffusible F (also called inorganic, exchangeable, or free F). It does not bind to serum proteins (3,19,20) and its fasting levels fall around the detection limit of the electrode. In this paper we use the term "diffusible" to identify the F in the serum ultrafiltrate. The latter contains ionic F and it may contain acid-labile, low-molecular weight, diffusible, F-containing compounds.

The second fraction is nondiffusible F (protein-bound F). It is not detectable by the electrode and for untreated subjects it has not yet been characterized in terms of their chemical nature, sources, biological significance or fate.

Together, the diffusible and nondiffusible fractions constitute the "total" serum F.

TABLE 2. Total Serum F: Precision Evaluation Experiments at Two Concentrations of F

	1 μM F	10 μM F
Single aliquot		
Within-run precision (Swr)	2.5	9.6
Total precision estimation (ST)	3.9	12.3
Coefficient of variation	393%	123%
Duplicate aliquots		
Within-run precision (Swr)	2.6	4.4
Total precision estimation (ST)	2.9	5.1
Coefficient of variation	288%	51%
Triplicate aliquots		
Within-run precision (Swr)	1.1	3.8
Total precision estimation (ST)	1.4	4.7
Coefficient of variation	138%	47%
Quadruplicate aliquots		
Within-run precision (Swr)	0.6	2.2
Total precision estimation (ST)	0.6	2.6
Coefficient of variation	63%	27%

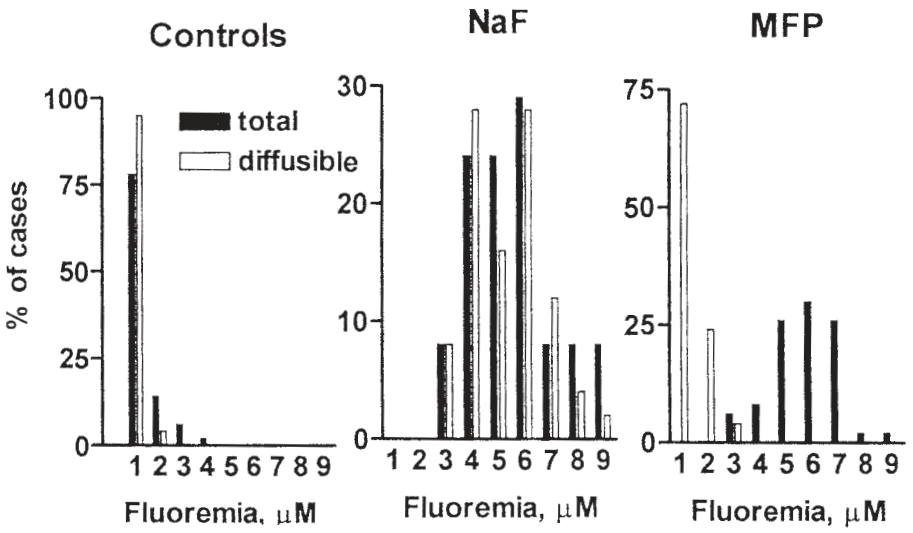

Fig. 4. Total (solid bars) and diffusible F (open bars) of fasting postmenopausal untreated women (left panel, n = 50), from 25 women receiving 30 mg of F/day (as NaF) for 6 to 14 months (center panel) and from 50 women treated with 15 mg of F/day (as sodium monofluorophosphate, right panel). The figures of the abcissa indicate the upper limit of each class width (e.g., 1 = 0.1–1.0 μM, 2 = 1.1–2.0 μM, etc.).

Isolation of serum diffusible F by means of an ion exchange resin (21) or by adsorption on calcium phosphate (5) have been reported, and confirmed the presence of diffusible and protein-bound F in serum. These techniques have not been extensively used. The availability of disposable ultrafiltration membranes, made this fast, clean and inexpensive analytical device the choice for separating diffusible F. The Taves' distillation procedure (11) provides three further analytical advantages: a fivefold (or greater) increase in the concentration of F in the measuring solution (with respect to that of serum or serum ultrafiltrate), the simplicity of the distillate composition, and the similarity with that of standards.

We routinely assay each serum sample or ultrafiltrate by quadruplicate. The isothermal distillation of F, though a very simple procedure, requires several days to attain completeness. The latter feature is often regarded as a negative one; but this way, a large number of samples can be handled simultaneously. The proposed technique has been used extensively in experimental and clinical work (7–9).

Accuracy and Precision of the Proposed Technique

Accuracy and precision statements in clinical reports show a large degree of ambiguity. From mere "standard methods" (22), passing through "sera were measured using a specific F ion-sensitive electrode" (23) to "F concentration in serum were performed by laboratories located in four local universities

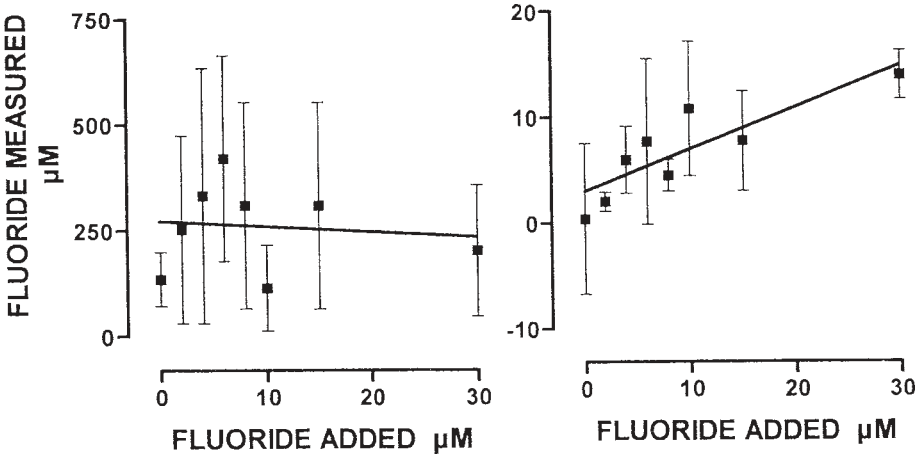

Fig. 5. Recovery of F from dialyzed serum according to Fry and Taves' method (left panel) (25) and to Fuchs et al. (right panel) (26).

laboratories using ion-specific potentiometry and were quality controlled" (24).

The Fry and Taves' technique (25) was published without precision or accuracy assessments. This technique greatly overestimates the F content with a large variance (Y-intercept: 273 ± 99 μM, Slope: –1.25 ± 7.7, r = 0.133, $P = 0.460$) and is, therefore, unsuitable for clinical analysis (Fig. 5, left panel). Fuchs et al. (26) claim a within-batch precision of (VC) 6.7% and 1.8% at 0.5 and 1.8 μM F. The Fuchs et al. technique produced a Y-intercept of 3.0 ± 2.3 μM, a slope 0.418 ± 0.18 (implicating an average 41.8% recovery) and poor sensitivity (the slope is significantly lower than 1.0), r = 0.43, $P = 0.035$)(Fig. 5, right panel).

The technique described here requires four replicates of each sample to reduce imprecision to acceptable levels (Table 2). The reader should note that the objective sought in measuring serum F is to monitor F administration avoiding toxic levels of the anion. Table 2 indicates that precision increases as a function of the number of replicates.

Fasting Serum F of Controls and of Patients Chronically Treated With Sodium F or Sodium Monofluorophosphate

Published fasting levels of serum F show a large range caused by differences in analytical technique. Renal function (27,28) and parathyroid status of subjects (28) are significant determinants. Maintaining all these factors constant, the fasting levels of serum F are a function of F exposure. According to Ekstrand (29), communities with tap water supply containing 0.5, 1.23, and 9.6 ppm (26, 65 and 505 μM, respectively), had fasting serum levels averaging 0.6, 1.0, and 1.7 μM. In agreement with other reports (17,18), subjects with a low spontaneous intake of F show a concentration of the nondiffusible form that is greater than that of diffusible F. Perusal of Figure 4 reveals that the F levels (total and diffusible) of normal untreated subjects do not have a gaussian distribution. This is not surprising for an anion that is adventitial to humans, with no known metabolic control. A similar conclusion was reached by Cowell and Taylor (n = 497) (30) but not by others (Husdan et al., n = 136; Singer and Orphaug, n = 225) (31,32).

Though several reports have been published on the F content of human serum, reference values should be obtained locally, attending the F content of drinking water, renal function, etc., as stated above. The upper 75 percentile of the controls values is proposed as a realistic cut-off point.

As expected, treatment with NaF increases the fasting levels of total and diffusible F for patients treated with sodium monofluorophosphate. On the other hand, the raised serum F levels are due to the increased protein-bound fraction, as reported elsewhere (7–9).

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