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Gas Chromatographic Method for the Determination of Fluoride Ion in Biological Samples. I. Fluoride Level in Monkey

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A gas chromatographic method for the assay of more than 5 ng/ml of fluoride in biological samples was established. By this method, the fluoride concentrations in monkey plasma, blood-corpuscles and urine were measured. The fluoride concentration in plasma, which was the lowest before a meal (13—21 ng/ml), increased with time after the ingestions of diet and water. The value reached about twice (22—37 ng/ml) the initial value. Although the fluoride levels before and after a meal differed among animals, the level of each individual monkey was similar to that observed again a week later. The fluoride level in monkey blood-corpuscles was about half the plasma level. Total fluoride recovered in urine for 24 h amounted to 77 to 219 μg. The excreted amounts of fluoride, although they were different among the individuals, were similar in each individual to that observed again after a week. Fluorides in plasma and urine of rat and human were also determined, and compared with those of monkey. The control levels of fluoride in experimental animals determined in this study represent useful basic data for the determination of fluoride produced by metabolism of fluorine-containing drugs.

Keywords——fluoride ion; gas chromatography; cynomolgus monkey; rat; plasma; blood-corpuscle; urine; trimethylchlorosilane; trimethylfluorosilane; animal feed

Fluoride is not toxic in humans unless an excessive amount is ingested. Endogenous fluoride levels in humans are more than 1000 ppm in bone, and of the order of 10^{-9} and 10^{-6} g/ml in plasma and urine, respectively. When people take water from a supply containing a high concentration of fluoride for a long period, they suffer from fluorosis, such as mottled teeth and osteosclerosis.[1,2] Methoxyflurane,[3] previously used as an anesthetic, produces a high plasma level of free fluoride on metabolism, and is currently out of use because of its nephrotoxicity. It is crucial from the viewpoint of drug safety to know whether and at what level fluoride is formed *in vivo* on metabolism after administration of fluorine-containing drugs. The endogenous fluoride level in humans is known in detail,[4,5] but few data in monkeys have been reported. The purpose of our study was to establish an assay method suitable for biological samples and to evaluate endogenous fluoride levels in monkeys as basic data for toxicological studies.

Gas chromatography (GC) has higher sensitivity than other methods, and is suited for the assay of nanogram order of fluoride concentration in plasma and blood-corpuscles. In order to apply GC to biological samples, we improved the assay method established by Fresen *et al.*[6] or Glerum *et al.*[7] The derivatization of fluoride is shown as follows:

$$(CH_3)_3SiCl + H_2O \longrightarrow (CH_3)_3SiOH + HCl$$

$$(CH_3)_3SiOH + H^+ + F^- \longrightarrow (CH_3)_3SiF + H_2O$$

This paper describes the determination of fluoride levels in cynomolgus monkey's plasma, blood-corpuscles and urine and the evaluation of their daily fluctuation, as well as the quantitative change after diet intake by using our improved GC method. The same method was also applied to human and rat samples and the fluoride levels found were compared with those in the case of the monkey.

Experimental

Apparatus——An Iwaki KM shaker, type V-S, was used for mixing and extraction, a Kubota centrifuge, model KR-600P, for separation.

GC: A Shimadzu, model GC-7A, equipped with a flame ionization detector was used.

Column: A glass column (2.6 m × 3 mm i.d.) was packed with 12% OV-101 (Applied Science Laboratories Inc.) coated on Chromosorb W (HP) (80/100 mesh, Wako Pure Chemical Industries Ltd.). The glass was silanized with a benzene solution containing 1% each of *N*-trimethylsilylimidazole, trimethylchlorosilane (TMCS) and *N,O*-bis(trimethylsilyl)acetamide.

GC Conditions: The carrier gas was nitrogen at a rate of 20 ml/min. The pressures of air and hydrogen were 0.5 and 0.6 kg/cm^2, respectively. The temperatures of the column oven, the injection port and the detector were 60, 160 and 160 °C, respectively. The sensitivity was set in the range of 10.

Data Integration System: A Shimadzu Chromatopac C-R1A data processor was used (ATTEN 2). The chart speed was 5 mm/min.

GC/Mass Spectrometry (MS): GC/MS was carried out using a Hitachi M-68 gas chromatograph/mass spectrometer (ion accelerating voltage, 3 kV; ionizing current, 115 μA; ionization chamber temperature, 120 or 140 °C) linked to a Hitachi 003 data system.

Column: The packing material was the same as used in GC (column, 1m × 3mm i.d.).

GC/MS Conditions: The flow rate of helium used as a carrier gas was 15 ml/min. The temperatures of the column oven, the injection port and helium separator were all 50 °C.

Reagents and Materials——2% (v/v) TMCS Toluene Solution: Pipet 0.5 ml of TMCS (Gasukuro Kogyo Co., Ltd.) into a 25-ml volumetric flask and dilute to the mark with toluene (Nakarai Chemicals Ltd., reagent for liquid scintillation counting).

3 N $HClO_4$: Pipet 162 ml of 60% perchloric acid (Kanto Chemical Co., Inc.) into a 500-ml volumetric flask and dilute to the mark with H_2O. In order to avoid contamination of fluoride, water used for preparation of reagent and sample solutions was purified by distillation of deionized water. All the measuring vessels and implements were made of polypropylene, from which fluoride release was not detected.

Standard Solution of NaF: Accurately weigh about 2.2 mg of NaF (standard reagent for volumetric analysis; Hashimoto Chemical Industry Co., Ltd.) into a 100-ml volumetric flask, dissolve it in and dilute to the mark with H_2O. Prepare several solutions with required concentrations by diluting the stock solution with H_2O.[8]

Assay Procedure——Blood Sample: Pipet 1 ml of the sample into a 15-ml polypropylene centrifuge tube (12.5 × 1.4 cm i.d.) and add exactly 4 ml of H_2O, exactly 1 ml of 3 N $HClO_4$ and 3 ml of ethylene dichloride. Stopper the tube and shake for 10 min. After centrifugation at 3000 rpm for 10 min, transfer exactly 5 ml of the supernatant into a 15-ml centrifuge tube. Add exactly 0.5 ml of 2% (v/v) TMCS toluene solution. Stopper the tube and shake for 10 min. After centrifugation at 3000 rpm for 10 min, inject 5 μl of the toluene layer into the column and proceed under the above gas chromatographic conditions. Measure the peak height of trimethylfluorosilane (TMFS). Calculate the fluoride concentration from the calibration curve according to the equation.

$$\text{fluoride concentration } (\mu\text{g/ml}) = \frac{\text{found concentration } (\mu\text{g/ml}) \times 1.2 \times 100}{\text{recovery } (\%)}$$

Recovery values (%) were 88.1 for human plasma, 91.9 for monkey plasma, 97.2 for monkey blood-corpuscles and 95.3 for rat plasma.

Urine: Pipet 1 ml of urine into a 15-ml centrifuge tube and add exactly 4 ml of H_2O, 1 ml of 3 N $HClO_4$ and 0.5 ml of 2%(v/v) TMCS toluene solution. Stopper the tube and shake for 10 min. After centrifugation at 3000 rpm for 10 min, inject 5 μl of the toluene layer into the column and proceed under the above GC conditions. Measure the peak height of TMFS. Calculate the fluoride concentration from the calibration curve.

Calibration Curve: Use 1 ml each of standard solutions. Proceed as directed in the urine procedure. Make a calibration curve by plotting the peak heights of TMFS against the concentrations of the standard solutions. Use the calibration curve for both plasma and urine analyses.

Examination of the Assay Conditions——The effect of TMCS concentration on the derivatization in the presence of serum was examined. The fluoride standard solution (1 μg/ml; 1 ml) and H_2O (3 ml) were added to 1 ml of human

control serum (Flow Lab., type AB). Then 3 N $HClO_4$ (1 ml) and ethylene dichloride (3 ml) were added and the mixture was shaken for 10 min. After centrifugation, the supernatant (5 ml) was transferred and TMCS toluene solution (1 to 5% (v/v), 0.5 ml each) was added. The mixture was assayed according to the assay procedure. The peak height of TMFS was measured.

Recovery Test of the Assay Procedure——Blood Sample: A fluoride standard solution (0.01 to 1 μg/ml; 1 ml) and H_2O (3 ml) were added to 1 ml of plasma (or blood-corpuscles). Then ethylene dichloride (3 ml) and 3 N $HClO_4$ (1 ml) were added. The mixture was assayed according to the assay procedure. A blood sample alone was assayed separately (blank). The peak height of TMFS was measured. The fluoride concentration recovered was obtained by subtracting the blank value from the found value.

Urine: A fluoride standard solution (0.1 to 1.5 μg/ml; 1 ml), H_2O (3 ml) and 3 N $HClO_4$ (1 ml) were added to 1 ml of urine. The mixture was assayed according to the assay procedure. Urine alone was assayed separately (blank). The peak height of TMFS was measured. The fluoride concentration recovered was obtained by subtracting the blank value from the found value.

Animal Experiment——Female cynomolgus monkeys weighing 2.2 to 3.4 kg were used. They were bred under ordinary conditions, and allowed free daily access to 100 g of a commercial diet (Oriental Yeast Co., Ltd., primate diet, type AB) and 70 g of cabbage. The food was put in at 11 a.m. and taken out at 5 p.m. every day. Animals were permitted free access to water all the time. Blood samples (2.5 ml) were collected at the specified time before and after a meal from a hind limb vein by using a Terumo disposable syringe in which 50 μl of heparin sodium for injection (1000 unit/ml, Shimizu Pharmaceutical Co., Ltd.) had previously been placed. After centrifugation at 3000 rpm at 4 °C for 15 min, separated plasma was placed in a polypropylene tube and stored in a refrigerator at −80 °C until the assay. Spontaneously excreted urine was collected overnight from the previous day to the experimental day and at the specified time intervals before and after a meal. The urine sample was placed in a polypropylene tube and stored at −80 °C.

Male and female, eleven-week-old, Jcl:SD rats were used. Feeding was stopped overnight before the assay. Water (water supply) was given freely overnight. The urine was collected for 4 h in the cage without a supply of water. The rats were anesthetized with ether, and blood (5 ml) was collected from the inferior vena cava using a heparin-containing syringe. Plasma was separated and stored in the same manner as described above. The urine sample was also stored similarly.

Results and Discussion

Assay Conditions

Selection of Solvent——The derivatized TMFS was extracted with solvent, then assayed by GC. TMFS was eluted at the retention time of 1.57 min and then the solvent was eluted (20—60 min). A suitable solvent for the extraction of TMFS is required to have no interfering peaks around the retention time of TMFS, and the retention time of the solvent alone should be as fast as possible to shorten the analysis time. Carbon tetrachloride, used by Glerum *et al.*,[7] could not be used because of the interfering peaks, which are shown in Fig. 1. Benzene, used by Fresen *et al.*,[6] was not suited for the assay in spite of the absence of such peaks, because complete elution took too long (over 60 min). Among other ten solvents tested, toluene was selected as one of the most suitable (analysis time, 30 min).

Derivatization Conditions——Trimethylsilylation, despite a two-phase reaction of aqueous fluoride solution with TMCS in toluene, proceeded rapidly. Shaking the two layers for more than 10 min resulted in derivatization of fluoride in constant yield. More than 1% TMCS was enough to complete the derivatization.

Clean-Up of Blood Samples——When the derivatization was done by directly adding TMCS in toluene (0.5 ml) to the acidic solutions of plasma and blood-corpuscles, then shaking the mixture, the toluene layer was not separated clearly because of emulsion formation. The blood sample solution was shaken with ethylene dichloride in the presence of $HClO_4$. No interfering peak was observed on the chromatogram. Lipophilic components in samples and pigment from blood-corpuscles were transferred to the ethylene dichloride layer. When the aqueous layer was separated and then shaken with TMCS dissolved in toluene, isolation of the toluene layer was complete.

Determination of Recovery Rate——Various concentrations (0.01—1.0 μg/ml (as F^-)) of NaF spiked in blood samples were assayed. The relationship between the recovered and the

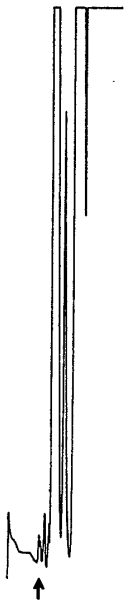

Fig. 1. Gas Chromatogram of Commercial Carbon Tetrachloride
↑, retention time of TMFS.

TABLE I. Recoveries of Fluoride from Biological Samples

Sample	Regression equation X=added Y=found	n	$s^{a)}$
Monkey plasma	$Y=0.923X-0.0024$	18	0.0121
Rat plasma	$Y=0.960X-0.0049$	28	0.0164
Human control serum	$Y=0.876X+0.0017$	18	0.0094
Monkey blood-corpuscles	$Y=0.974X-0.0015$	27	0.0101

a) Estimate of residual standard deviation.

added amounts was linear and the line passed through the origin. Table I shows the regression equations. From the slope of the straight line, the recoveries were calculated to be 91.9% for monkey plasma, 95.3% for rat plasma, 88.1% for human control serum and 97.2% for monkey blood-corpuscles. These recovery values measured in inter-day analyses were reproducible.

In the case of urine samples (monkey and human), fluoride was quantitatively recovered.

Fluoride in Monkey Plasma and Urine

Female cynomolgus monkeys which were bred under ordinary conditions were used. Blood and urine were collected at the specified time intervals before and after a meal, and were assayed. The results of GC analyses of the samples are shown in Fig. 2.

Effect of Diet and Water on Fluoride Concentration——Plasma Level: As shown in Fig. 3, fluoride concentrations in plasma were lower before a meal (13—21 ng/ml) than those examined at any time after a meal. The concentrations increased with time after the supply of diet to reach the maximum value (22—37 ng/ml) at 4—8 h. This shows that fluoride in plasma increases after ingestions of diet and water. Fluoride levels in the commercial diet and water supply were assayed to be 16—18 ppm and 0.08—0.1 ppm, respectively.[9] The amounts of diet and water ingested varied among individuals (5—100 g (mean, 52 g) and 62—470 ml (mean, 190 ml)) and they also varied between the two experimental days. The increasing fluoride concentrations after a meal were not directly associated with the amounts of diet[10] and water ingested. The amounts of diet and water were measured as total values ingested over 6 h. Since the eating and drinking speeds were different among the individuals, the absorbed amounts contributing to the fluoride level at the measuring time were not clear. The absence of

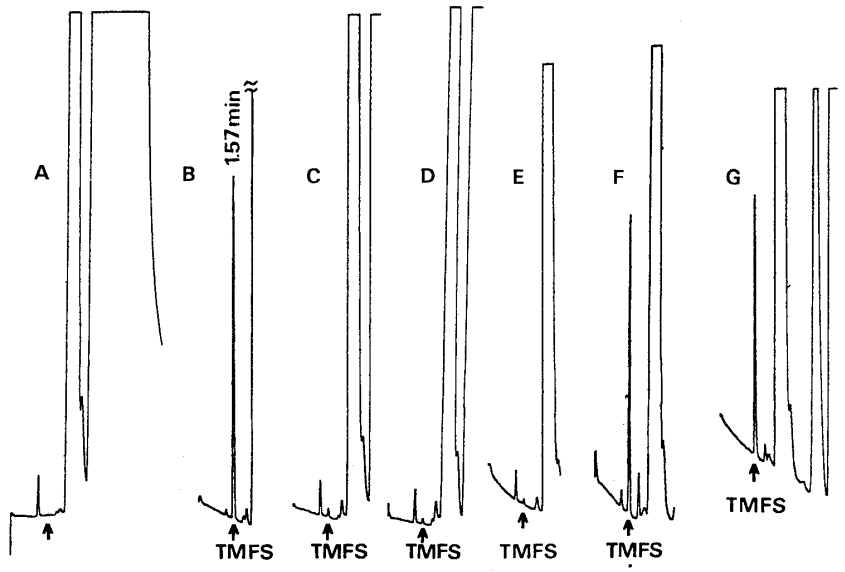

Fig. 2. Gas Chromatograms Obtained through Assays of Standard Solution and Biological Samples

A) Reagent blank. B) Standard solution of fluoride ion (0.5 μg/ml). C) Monkey plasma. D) Monkey blood-corpuscles. E) Human plasma. F) Monkey urine. G) Human urine.

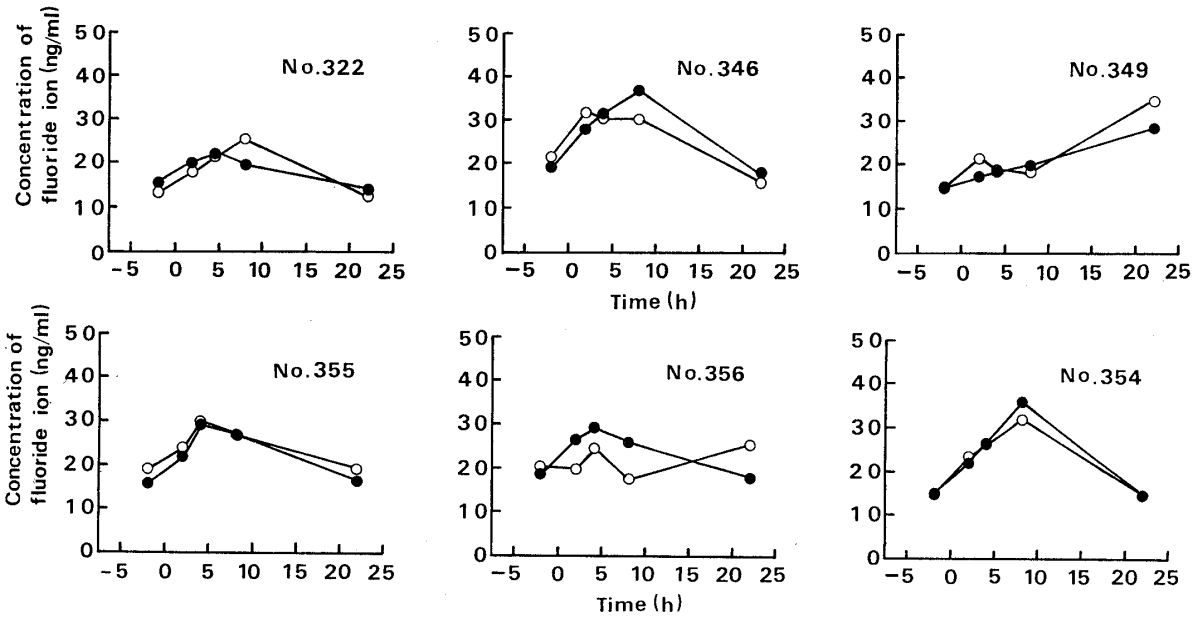

Fig. 3. Plasma Levels of Fluoride Ion in Monkeys Fed in the Ordinary Life Cycle

Time 0, supply of food; —●—, exp. 1; —○—, exp. 2.

apparent dependence of fluoride levels on the total amounts of diet and water may be due to the difference in the ingesting speed or absorption rate of diet among the individuals.

After the maximum, fluoride concentration decreased and returned to the levels before the meal after 22 h, although one individual (No. 349) exceptionally showed a continuing increase of fluoride. A large difference in the ingested amounts of diet and water between No. 349 and others was found: the amounts of diet and water taken by No. 349 were very much larger (80 g and 300 ml, and 100 g and 470 ml, on the first and second experimental days, respectively) than those taken by the others.

Urine Level: Figure 4 shows the hourly amounts of fluoride excreted in urine for 24 h after a meal, together with those recovered before the meal (collected for 14 h (overnight) and

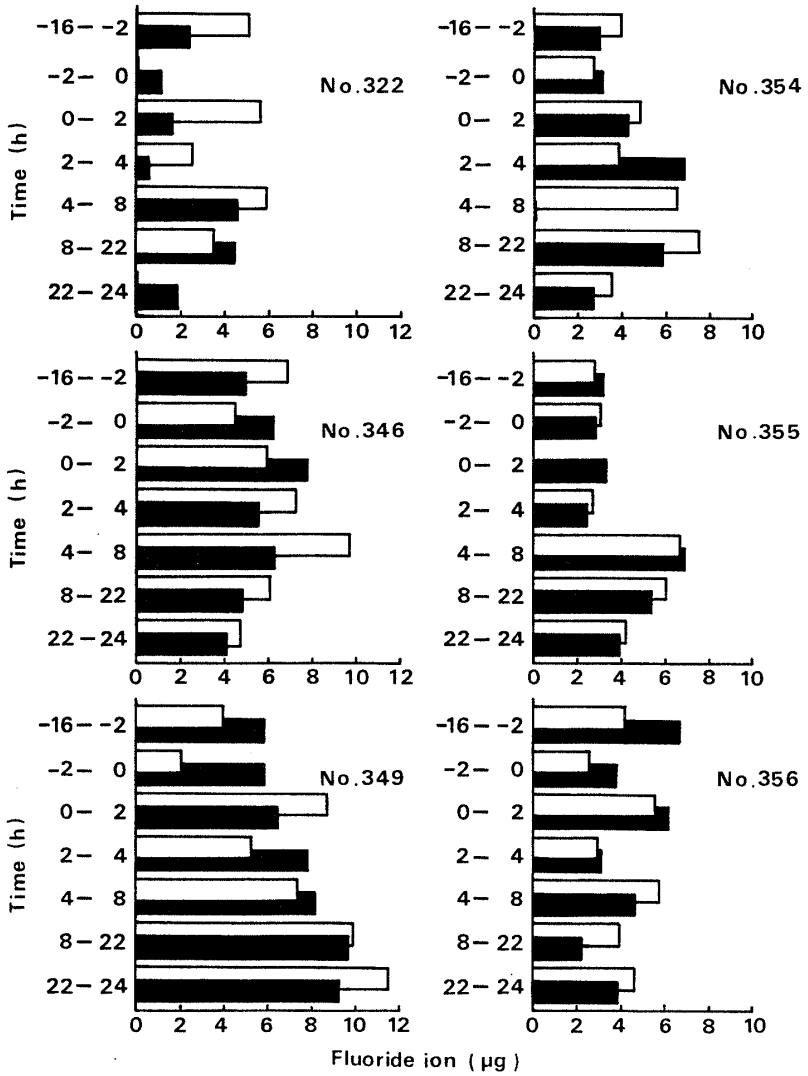

Fig. 4. Amount of Fluoride Ion Excreted in Monkey Urine per Hour
Time 0, supply of food; □, exp. 1; ■, exp. 2.

2 h). The values are expressed as the excretion velocity (fluoride content (μg) per hour). The rate of excretion varied greatly among the individuals. Even in the same monkey, the values for the two time intervals before the meal (-16——-2 h and -2—0 h) were different from those observed at the same time on the following day (8—22 h and 22—24 h). There were some individuals whose rates of excretion increased after ingestions of diet and water, while some cases showed no such effect. Total fluoride recovered after a meal amounted to 77 to 219 μg (mean, 133 μg). The quantities varied greatly among the individuals. No relationship was observed between the amounts of excreted fluoride and the diet taken by the animal. However, there was a tendency for the amount of fluoride to increase with increasing intake of water supply, resulting in a rough correlation ($r=0.83$, $n=12$) between the amounts of fluoride and water supply. In fact, No. 349 which drank the greatest amount of water showed the highest recovery (219 and 216 μg on the two experimental days) of all the monkeys tested.

Effect of Day on Fluoride Concentration——Plasma Level: Plasma was collected after a week from the same monkeys and fluoride was assayed again. As shown in Fig. 3 (exp. 2), the levels before and after a meal were similar to those found a week previously. Although most individuals ingested different amounts of diet and water before and after a week, fluoride in each monkey's plasma remained at almost the same level.

TABLE II. Analytical Results of Fluoride in Biological Samples

Sample[a]		Fluoride (μg/ml) $\bar{x}\pm$S.D.	n
Plasma	Human[b]	0.0103±0.0032	(9)
	Monkey[c]	0.0173±0.0028	(5)
	Rat[c]	0.0529±0.010	(4)
Urine	Human[d]	0.528 ±0.218	(18)
	Monkey[e]	0.156 ±0.055	(5)
	Rat[f]	7.44 ±1.72	(4)
Blood-corpuscles	Monkey[c]	0.0095±0.0040	(5)

a) Monkey and rat samples were collected from the same individuals. *b*) Collected after breakfast. *c*) Collected in the fasting state from the previous day. *d*) Collected at various times of day. *d*) Collected at various times of day. *e*) Collected for 2 h in the fasting state from the previous day. *f*) Collected for 4 h (without supply of water) in the fasting state from the previous day.

Urine Level: Urine assay was simultaneously carried out. As shown in Fig. 4 (exp. 2), hourly amounts of fluoride excreted from each animal were different from those observed a week previously. However, the total amounts of fluoride excreted over 24 h were similar between the two dates. In this case there was also a tendency that the greater the amount of water the animal took, the more fluoride it excreted.

Fluoride in Monkey Blood-Corpuscles

Fluoride levels in plasma and blood-corpuscles were assayed at the same time on a different day from the above experimental days. Fluoride in blood-corpuscles of five monkeys in the fasting state was 9.5 ng/ml (mean, $n=5$) (Table II). The concentrations were much lower (about half) than those in plasma (mean, 17.3 ng/ml, $n=5$).

Comparison of Fluoride Level in Monkeys with Those of Humans and Rats

The fluoride level in human plasma of healthy males was 10.3±3 ng/ml, as shown in Table II. The plasma samples were collected about 1 h after breakfast. The level was in good accord with that reported by Yoshida *et al.*,[5] the average fluoride level of Japanese being 12±8 ng/ml. The level in monkeys, even in a fasting state where the fluoride level is the lowest in the day, is higher than that of humans. Rat plasma contained the highest concentration of fluoride, 52.9 ng/ml.

The fluoride level in human urine of healthy males collected at various times of day was 0.528±0.218 μg/ml. The values were similar to the average fluoride levels of Japanese measured by Yoshida *et al.*[5] (0.72±0.46 μg/ml). The levels in animals shown in Table II were assayed in the fasting state before a meal. Rats excreted urine containing the highest concentration of fluoride. Since the volume of the collected urine was very small, it seems likely that fluoride was concentrated in the fasting rat's urine.

Identification of TMFS by GC/MS

TMFS derivatized from monkey plasma, blood-corpuscles and urine as described for GC assay was identified using the GC/MS system. Each of the toluene extracts obtained from these samples was subjected to GC/MS. Each mass chromatogram showed a peak at 0.3 min (Scan No. 20), as shown in Fig. 5. Figure 6 shows the mass spectrum of this peak obtained from a urine sample. The spectrum showed a fragment ion of the highest intensity at m/z 77, corresponding to $(TMFS-CH_3)^+$ and a molecular ion (m/z 92) of a lower intensity, clearly identifying the peak substance as TMFS. Although the molecular ion peak was not detected in plasma and blood-corpuscle samples because of its lower sensitivity, the peak of $(TMFS-CH_3)^+$ was confirmed. Moreover, the mass chromatogram of the toluene extract from a

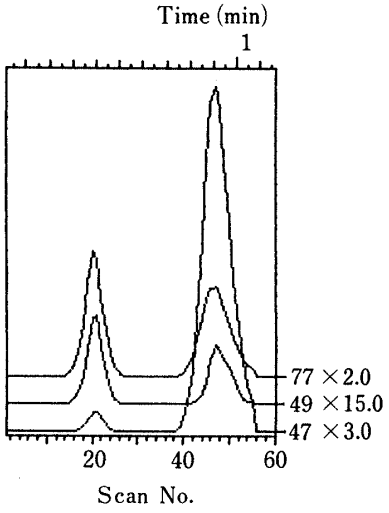

Fig. 5. Mass Chromatogram of TMFS Obtained from Monkey Urine

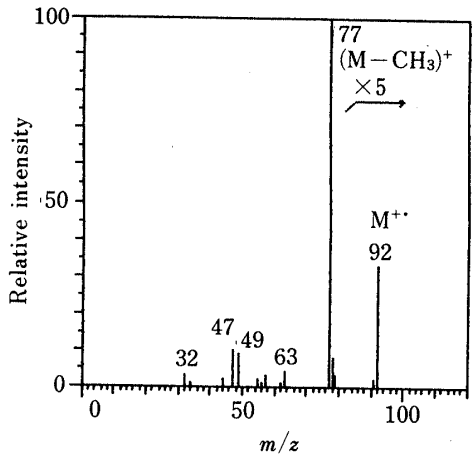

Fig. 6. Mass Spectrum of TMFS Obtained from Monkey Urine

standard NaF sample showed a base peak at 0.3 min and its mass spectrum was identical with that from each biological sample.

Conclusion

Fluoride in biological samples is assayable by using a GC method. According to our method, more than 5 ng/ml of fluoride in plasma, blood-corpuscles and urine[11] can be assayed with a coefficient of variation of less than 5%. In the present work the endogenous fluoride levels in monkey and rat were clarified. This represents basic data to evaluate fluoride produced *in vivo* after administration of a fluorine-containing drug. It was found that the fluoride level varies markedly within a day, depending on the ingestions of diet and water. It is thus advisable to administer a drug to fasted animals for the assay of fluoride.

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8) All concentrations in this paper refer to fluoride ion.
9) The assay method was applicable to animal feed samples. Suspensions of feed and cabbage in water were assayed according to the procedure for plasma. No difficulty in pretreatment and no interfering peaks were found.
10) Since fluoride content in cabbage (0.05 ppm) is much lower than in the solid diet, its influence on plasma level can be neglected.
11) Fluoride in urine is high enough to be measured by the ion-selective electrode (ISE) method. The fluoride concentrations in urine samples analyzed by this method were redetermined by using the ISE method.[5] There was a good correlation ($r=0.98$, $n=96$) between the values obtained in the two assays.