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## Gas Chromatographic Method for the Determination of Fluoride Ion in Biological Samples. II.<sup>1)</sup> Stability of Fluorine-Containing Drugs and Compounds in Human Plasma

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Our gas chromatographic method established previously has been modified to assay fluoride ion which is released from fluorine-containing compounds and drugs in human plasma. A strongly acidic medium was not necessarily required, though fluoride is usually derivatized to trimethylfluorosilane (TMFS) under such acidic conditions. Trimethylsilylation proceeded in pH 2.5 sulfosalicylate (SSA) buffer. Even labile fluorine-containing compounds were stable in SSA buffer, while they were degraded in a strong acid to form fluoride. SSA was effective for buffering HCl which was produced by the reaction of excess reagent, trimethylchlorosilane (TMCS), with H<sub>2</sub>O. At the same time, SSA in plasma sample served as not only a deproteinizing agent but also a buffer agent for biological materials. By means of the present method, released fluoride in human plasma is assayable even in the presence of labile fluorine-containing compounds. More than 5 ng/ml of fluoride can be quantitated.  $\alpha$ -Fluoro- $\beta$ -alanine, 4-fluoroglutamic acid, 3-difluoroalanine and 5-(trifluoromethyl)uracil released fluoride in human plasma at 37 °C *in vitro*. The amount of fluoride increased with time. On the other hand, no fluoride was liberated from 5-fluorouracil or from compounds or drugs studied other than the above ones.

**Keywords**—fluoride ion; gas chromatography; trimethylchlorosilane; trimethylfluorosilane; sulfosalicylate buffer; stability; fluorine-containing compound; 5-fluorouracil;  $\alpha$ -fluoro- $\beta$ -alanine; human plasma

Some clinically used fluorine-containing drugs release fluoride ion as a result of metabolism in humans.<sup>2)</sup> Since a large quantity of fluoride is toxic to humans, it is crucial from the viewpoint of drug safety to know whether or not fluorine-containing drugs would release fluoride in human plasma. Previously we established a gas chromatographic method to assay fluoride in plasma.<sup>1)</sup> Fluoride was derivatized to trimethylfluorosilane (TMFS) in a strong acid. However, the assay method, when applied to the stability assessment of fluorine-containing drugs, presented difficulty: some compounds were chemically degraded during the assay under the acidic conditions to produce fluoride, obscuring the estimation of biologically released fluoride in human plasma. We examined the effect of pH on the derivatization and found that fluoride in more weakly acidic medium at pH 2.5 was derivatized quantitatively. This paper describes a gas chromatographic method employing pH 2.5 sulfosalicylate (SSA) buffer and presents stability data on some fluorine-containing drugs and compounds in human plasma *in vitro*.

### Experimental

**Apparatus**—A Taiyo incubator, type M-1<sup>N</sup>, was used for incubation. The other apparatus were the same as described in the previous paper.<sup>1)</sup>

**Reagents and Materials**—pH 2.5 SSA Buffer: Dissolve 50.8 g of sulfosalicylic acid (GR-grade, Kanto Chemical Co., Inc.) in and dilute with H<sub>2</sub>O to make 200 ml, and then adjust the pH to 2.5 by adding 1 N NaOH (standard normal solution, Nakarai Chemicals Ltd.).

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, then dissolve it in and dilute to the mark with H<sub>2</sub>O. Prepare several solutions with required concentrations by diluting the stock solution with H<sub>2</sub>O.<sup>3)</sup>

The other reagents and the reagent solutions were the same as those used in the previous paper.<sup>1)</sup> 3-Difluoroalanine, 4-fluoroglutamic acid, 5,6-dihydro-5-fluorouracil and floxacillin sodium monohydrate were supplied by chemists of our laboratories. The other fluorine-containing compounds were obtained commercially (reagent grade).

**Stability Test of Fluorine-Containing Drugs and Compounds in Human Plasma and Saline**—One milligram each of the samples except for floxacillin sodium monohydrate and  $\alpha$ -fluorocinnamic acid was dissolved in 1 ml of saline (Hishiyama Pharm. Co., Ltd.). Each solution (110  $\mu$ l) was added to fresh human plasma (11 ml) with good mixing. To prepare higher concentrations of sample solutions, 2 mg of floxacillin sodium monohydrate was directly dissolved in fresh human plasma (10 ml).  $\alpha$ -Fluorocinnamic acid (20 mg) was dissolved in 1 N NaOH (0.5 ml) and then 1 N HCl was added to adjust the pH to about 8, followed by dilution with saline to make 2 ml. The solution (220  $\mu$ l) was added to fresh human plasma (11 ml) with good mixing. Each plasma sample solution (1 ml) was transferred to 15-ml polypropylene centrifuge tubes. The tubes were stoppered tightly and incubated in an incubator (73 times agitation/min) at 37 °C for 1 to 5 h. The samples were frozen and stored at -20 °C until assay. After thawing, these samples were analyzed according to the standard assay procedure.

Sample solutions of the same concentrations in saline only were also prepared and the stability was examined by the same method.

**Assay Procedure**—Pipet 1 ml of plasma sample into a 15-ml polypropylene centrifuge tube (12.5  $\times$  1.4 cm i.d.), and add exactly 5 ml of pH 2.5 SSA buffer 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 to a 15-ml centrifuge tube. Add exactly 0.5 ml of 2% (v/v) trimethylchlorosilane (TMCS) toluene solution, stopper the tube and shake for 20 min. After centrifugation at 3000 rpm for 10 min, place the tube in crushed dry-ice<sup>4)</sup> for 30 min. After thawing in cold water, recentrifuge at 3000 rpm for 5 min. Inject 5  $\mu$ l of the toluene layer into the column and proceed under the gas chromatographic conditions described in the previous paper.<sup>1)</sup> Measure the peak height of TMFS. Calculate the fluoride concentration from the calibration curve according to the equation:

$$\text{fluoride concentration } (\mu\text{g/ml}) = \frac{\text{found concentration } (\mu\text{g/ml}) \times 1.2 \times 100}{91.4}$$

(The recovery of fluoride from plasma is 91.4%.)

**Examination of pH-Effect**—To 1 ml of the fluoride standard solution (1  $\mu$ g/ml), 5 ml of SSA buffer (pH; 2.05—3.00) and 0.5 ml of 2% (v/v) TMCS toluene solution were added. The mixture was shaken for 30 min. The peak height of TMFS was measured in the same manner as described in the assay procedure. The same experiment was repeated using acetate buffer (pH: 0.98—4.05) in place of SSA buffer.

**Examination of Shaking Time**—To 1 ml of the fluoride standard solution (1  $\mu$ g/ml), 5 ml of acetate buffer (pH; 0.98—4.05) or SSA buffer (pH; 2.5) and 0.5 ml of 2% (v/v) TMCS toluene solution were added. The mixture was shaken for 10—60 min. The peak height of TMFS was measured in the same manner as described in the assay procedure.

**Recovery Test of the Assay Procedure**—To 1 ml of human control serum (Flow Lab., type AB), a fluoride standard solution (0.01 to 1  $\mu$ g/ml; 1 ml), SSA buffer (4 ml)<sup>5)</sup> and ethylene dichloride (3 ml) were added. The mixture was assayed according to the assay procedure. Separately, control serum alone was assayed (blank). The fluoride concentration recovered was obtained by subtracting the blank value from the found value.

**Effect of Fluorine-Containing Drugs and Compounds on the Assay Procedure**—5-Fluorouracil, 5,6-dihydro-5-fluorouracil,  $\alpha$ -fluoro- $\beta$ -alanine, 4-fluoroglutamic acid, 3-difluoroalanine, 5-(trifluoromethyl)uracil and 5-fluorocytosine were each dissolved in SSA buffer (2.5  $\mu$ g/ml each). Each sample solution (4 ml) was mixed with a fluoride standard solution (0.01 to 1  $\mu$ g/ml; 1 ml) and then with the control serum (1 ml). The mixture was assayed according to the assay procedure. The fluoride concentration recovered was obtained in a similar manner to that used in the recovery test.

Separately, the effect of fluorine-containing compounds on the previous method<sup>1)</sup> was tested. Aqueous solutions of floxacillin sodium monohydrate and  $\alpha$ -fluorocinnamic acid (ca. 200  $\mu$ g/ml each), and 4-fluoroglutamic acid,  $\alpha$ -fluoro- $\beta$ -alanine and 3-difluoroalanine (ca. 10  $\mu$ g/ml each) were used in this procedure. Each solution (1 ml) was mixed with a standard fluoride solution of NaF (0.01 to 1  $\mu$ g/ml; 1 ml), H<sub>2</sub>O (2 ml) and the control serum (1 ml). The mixture was assayed according to the assay procedure. The fluoride concentration recovered was obtained by subtracting the blank value from the found value.

## Results and Discussion

### Effect of pH on Derivatization

In order to examine the effect of acidity on trimethylsilylation of fluoride, fluoride in

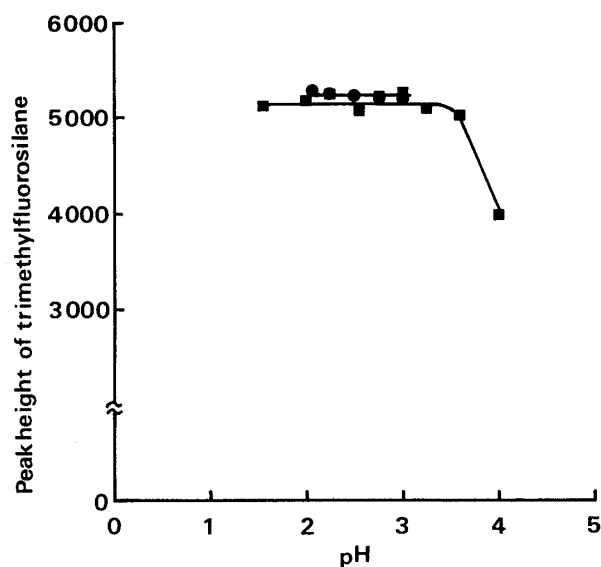


Fig. 1. Effects of pH on Trimethylsilylation  
 —●—, 1 M SSA buffer; —■—, 1 M acetate buffer.

buffers of various pH's (SSA and acetate) was derivatized. Figure 1 shows the peak heights of TMFS derivatized at pH 1.0 to 4.0. A constant peak height was obtained at pH lower than 3.6. The effect of shaking time on derivatization was preliminarily tested. A constant yield of TMFS at each pH was obtained by shaking for more than 10 min. The derivatization progressed completely even at around pH 3. SSA buffered HCl which was produced during the derivatization by reaction of excess TMCS with  $H_2O$ . The use of this buffer resulted in no pH change before and after the derivatization. SSA in plasma sample also served as not only a deproteinizing agent but also a buffer agent for biological materials. After the derivatization in a buffer at pH less than 2.5 in the presence of plasma (1 ml), the pH rise was within 0.2. On the other hand, acetate buffers (1 M) were too weak to buffer the resulting HCl. From the viewpoint of analytical convenience, a pH 2.5 SSA buffer was used as the derivatization medium in the new assay procedure.

#### Validation of the Assay Method

A modified method, in which 3 N  $HClO_4$  used in the previous method<sup>1)</sup> was exchanged for SSA buffer, was tested and its quantitative usefulness was validated as shown below.

**Stability Check of Fluorine-Containing Compounds**—NaF and fluorine-containing compounds listed in Table I were spiked with a commercial human control serum. According to the modified method, fluoride was recovered quantitatively without being influenced by the fluoride from degraded fluorine-containing compounds. However, when the serum samples spiked with  $\alpha$ -fluoro- $\beta$ -alanine and 3-difluoroalanine were assayed by using the previous method,<sup>1)</sup> high levels of fluoride were recovered compared to the added amounts, indicating that these compounds were degraded to form fluoride under the old assay conditions. Such acid-labile fluorine-containing compounds were stable under the new assay conditions of the modified method.

**Recovery Test**—NaF was spiked with a commercial human control serum. Seven spiked solutions ranging from 0.01 to 1.0  $\mu g/ml$  as  $F^-$  were analyzed by the modified method. From the slope of the straight line drawn by using the found values and added values of fluoride, the recovery was calculated to be 91.4% ( $Y=0.914X$ ,  $s=0.0218$ ,  $n=25$ ). No difference in recoveries was observed through the interday analyses.

**Calibration Curve and Quantitation Limit**—The calibration curve of fluoride constructed under the new assay conditions was in good accord with that obtained by using the previous method.<sup>1)</sup> The concentration range over which the relationship was linear was 0.01 to 30  $\mu g/ml$ . The limit of quantitation was 5 ng/ml.

TABLE I. Stability of Fluorine-Containing Compounds and Drugs in Human Plasma

Compound	Concen- tration ( $\mu\text{g/ml}$ )	Sample	Concentration of fluoride ion ( $\mu\text{g/ml}$ )					(%) <sup>a)</sup>	Assay method
			Incubation time (h)						
			0	1	2	3	5		
5-Fluorouracil	9.90	Saline	0	0	0	0	0	SSA <sup>b)</sup>	
	10.43	Plasma	0	0	0	0	0		
5,6-Dihydro-5-fluorouracil	10.4	Saline	0	0	0	0	0	SSA <sup>b)</sup>	
	11.0	Plasma	0	0	0	0	0		
$\alpha$ -Fluoro- $\beta$ -alanine	10.8	Saline	0	0	0	0	0	SSA <sup>b)</sup>	
	10.1	Plasma	0	0.001 <sup>c)</sup>	0.003	0.005	0.005		(0.3)
4-Fluoroglutamic acid	12.6	Saline	0	0.001	0.002	0.002	0.004	(0.3)	SSA <sup>b)</sup>
	10.1	Plasma	0	0.008	0.018	0.024	0.041	(3.5)	
3-Difluoroalanine	9.60	Saline	0	0.001	0.001	0.001	0.006	(0.2)	SSA <sup>b)</sup>
	10.2	Plasma	0	0.004	0.020	0.027	0.041	(1.3)	
5-(Trifluoromethyl)uracil	11.3	Saline	0	0.015	0.030	0.042	0.065	(1.8)	SSA <sup>b)</sup>
	12.7	Plasma	0	0.053	0.116	0.166	0.290	(7.2)	
5-Fluorocytosine	12.3	Plasma	0	0	0	0	0	SSA <sup>b)</sup>	
Floxacin sodium monohydrate	210	Plasma	0	0	0	0	0	HClO <sub>4</sub> <sup>d)</sup>	
$\alpha$ -Fluorocinnamic acid	190	Plasma	0	0	0	0	0	HClO <sub>4</sub> <sup>d)</sup>	

a) Percent released of total amount of F in a molecule. b) Present method. c) An average value of duplicate runs. d) Previous method.

### Stability of Fluorine-Containing Compounds and Drugs in Human Plasma

Nine fluorine-containing compounds and drugs spiked with fresh human plasma were incubated in a water bath at 37°C. After the specified time of incubation, samples were assayed. Saline solutions were treated in the same manner to give controls. Table I shows that  $\alpha$ -fluoro- $\beta$ -alanine, 4-fluoroglutamic acid, 3-difluoroalanine and 5-(trifluoromethyl)uracil were not stable in human plasma. The fluoride concentration increased with the incubation time. The most fluoride was released from 5-(trifluoromethyl)uracil, amounting to 7.2% of its fluoride content.  $\alpha$ -Fluoro- $\beta$ -alanine, which released fluoride in plasma, was stable in saline. 4-Fluoroglutamic acid, 3-difluoroalanine and 5-(trifluoromethyl)uracil liberated a small amount of fluoride even in saline. The amounts of fluoride released in plasma were much more than those found in saline. The other fluorinated compounds and drugs were stable in both saline and plasma. Floxacin sodium monohydrate and  $\alpha$ -fluorocinnamic acid did not release fluoride in the strongly acidic medium of the previous method.<sup>1)</sup> No fluoride was released even in the presence of higher concentrations of these compounds (20 times the values of the other compounds).

Martino *et al.*<sup>6)</sup> have found fluoride in plasma of a patient intravenously administered with 5'-deoxy-5-fluorouridine (5'-dFUr). They suggested that fluoride was released from the final metabolite of 5'-dFUr,  $\alpha$ -fluoro- $\beta$ -alanine. Our *in vitro* stability data also suggest the likelihood of fluoride release from  $\alpha$ -fluoro- $\beta$ -alanine *in vivo*, but not from 5,6-dihydro-5-fluorouracil or 5-fluorouracil.<sup>7)</sup>

### Conclusion

Fluoride released from a fluorine-containing drug in human plasma could be exactly quantitated by improving the pretreatment method of our previous method. By this method, since labile fluorinated compounds in a strong acid were stable, stability assessment of a wider range of compounds and drugs should be possible, as compared with an ordinary method, as

long as they are stable at pH 2.5. Moreover, this method is applicable to urine samples and thus it may also be useful for stability assessment of such compounds in urine.

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#### References and Notes

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- 2) L. Singer and R. Ophaug, *CRC Crit. Rev. Clin. Lab. Sci.*, **18**, 111 (1983).
- 3) Concentrations in this paper are given as fluoride.
- 4) This procedure made the boundary surface between the toluene and buffer layer clean and facilitated sampling.
- 5) No pH change was observed between 4 ml and 5 ml of SSA buffer used.
- 6) R. Martino, A. Lopez, M. C. Malet-Martino, J. Bernadou, and J. P. Armand, *Drug Metab. Dispos.*, **13**, 116 (1985).
- 7) In ref. 6, it was shown that 5'-dFUr is degraded by metabolism to 5-fluorouracil, to 5,6-dihydro-5-fluorouracil and finally *via* two metabolites to  $\alpha$ -fluoro- $\beta$ -alanine.