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Determination of Fluoride Ion in Femurs of Rats Receiving Organic Fluoride

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With the introduction of phenothiazine compounds containing the trifluoromethyl group as tranquillizing agents, it was deemed necessary to ascertain the *in vivo* stability of the trifluoromethyl group. It was postulated that, if the fluorine atoms were liberated from the trifluoromethyl group within the body, the fluoride content of the bone would be increased.

There are many methods in the literature for the determination of fluoride in biological media. Numerous investigators have employed the Willard and Winter[1] perchloric acid or sulphuric acid distillation procedure for the separation of fluoride ion from interfering substances, and many modifications of this procedure have been described. However, only a few methods apply specifically to the determination of fluoride in bone.

Scott and Henne[2] ashed bones in a muffle furnace at 650–700° for 12 h without a fixative and, employing the Willard and Winter distillation procedure, determined the fluoride content by titration with cerous or thorium nitrate. Godfrey and Shrewsbury[3] ashed bone specimens with magnesium acetate at 570° for 6 h, distilled the fluoride as hydrofluosilicic acid and then employed the back titration technique of Dahle, Bonnar and Wichmann.[4] Jackson *et al.*[5] autoclaved beef, pork and veal bones prior to the ashing, distillation and subsequent titration of the fluoride ion. Muhler[6] determined the fluoride content of rat bones using the method of Smith and Gardner,[7] which involves a double distillation of the fluoride ion with subsequent titration with thorium nitrate. In all these procedures the fluoride ion is eventually titrated; the end-point, which is indistinct, depends upon the colour perception of the individual analyst.

In this laboratory a spectrophotometric method for the determination of fluoride ion in the bones of experimental animals was developed through the combination and modification of several published procedures.[1, 8, 9] The basic steps include ashing with slaked calcium hydroxide in platinum crucibles in a muffle furnace at 500° for 6 h, steam distillation of the fluoride ion from perchloric acid as hydrofluosilicic acid, treatment of the distillate with zirconium alizarin sulphonate reagent, and spectrophotometric measurement of the subsequent bleaching of the zirconium–alizarin lake by fluoride at 525 mμ.

Since the development of this method, Samachson, Slovik and Sobel[10] have published a micro procedure for the determination of fluoride in bone fragments using a modified alizarin reagent for the spectrophotometric determination of distilled fluoride. The new reagent, lanthanum chloranilate,[11] was not investigated, as the experimental phases of this work were completed before this reagent was available commercially.

In two studies SKF No. 5019–A_2, 10-[3-(1-methyl-4-piperazinyl)-propyl]-2-trifluoromethyl phenothiazine dihydrochloride, was administered to rats at dosage levels of 1·0 mg/kg day and 10 mg/kg day for 182 days and 75 days, respectively. After the animals were sacrificed, the femurs from the control and test animals were analysed for fluoride content. An increase in fluoride content in the femurs of the test animals over that found in the controls would be suggestive of *in vivo* instability of the trifluoromethyl group on the phenothiazine nucleus.

Procedure

A. Bone Specimens

Bone specimens, freed of tissue and bone marrow, weighing between 0·1 and 1·0 g, are dried at 120° for 72 h and stored in a desiccator.

Weigh the dried specimens and transfer to a 50-ml platinum crucible. Add 25 ml of the slaked calcium hydroxide suspension,[12*] evaporate to dryness at 120° and then ash in a muffle furnace at 500° for 6 h. Transfer each residue to a 250-ml, 3-neck distillation flask (24/40 joints) using a polyethylene policeman and three 10-ml portions of de-ionized water and two 20-ml portions of 70–72 per cent perchloric acid to effect the transfer. Add 6–8 glass beads and 5 g of silver perchlorate[13] to each flask.

* 70–72 per cent perchloric acid was used in place of 60 per cent.

Place each 3-neck flask in a heating mantle, and insert the teflon rod gland* containing the thermometer into the middle neck of the flask, immersing the tip of the thermometer beneath the surface of the liquid. To one side neck connect a Claisen distilling head (the main tube having been lengthened to 10 cm to prevent entrainment) which, in turn, is connected to a condenser by means of a 105° angle adapter. Into the other side neck insert the steam inlet tube which, in turn, is connected to the steam generator.[9]

Maintain the contents of the flasks between 134°–137° and steam distill the fluoride as hydrofluosilicic acid. Collect approximately 198 ml of distillate in a 200-ml volumetric flask containing 2 drops of 1 N sodium hydroxide.

Adjust the pH of the distillates to approximately 5 with 1 N hydrochloric acid and/or 1 N sodium hydroxide, and then bring the flasks to volume with de-ionized water. After shaking, pipette 100 ml of each distillate into 125-ml glass-stoppered Erlenmeyer flasks.

For the reference standard, pipette 100 ml of a 5 μg/ml standard into another 125-ml Erlenmeyer flask.

Add 10 ml of the diluted zirconium alizarin sulphonate reagent[8] to each flask, mix well, and allow to stand for 3 h. Measure the absorbance of the test solutions against the reference standard in a Beckman DU Spectrophotometer at 525 mμ, 0·06 slit width, with cells having a light path of 20 mm. The concentration of fluoride in 100 ml of the test distillates is then read from a standard curve.

If the concentration of fluoride in the distillate exceeds the limits of the standard curve, a smaller aliquot of the distillate should be taken and diluted to 100 ml for colour development.

B. Standard Curve

Prepare a series of standard solutions containing 50, 100, 150, 200, 250 and 300 μg of fluoride per ml of solution from a stock fluoride solution. Pipette 1 ml of each of the diluted standards into platinum crucibles followed by 25 ml of the calcium hydroxide suspension. Then treat the standards in exactly the same manner as the bone specimens, including the 6-h heating in the muffle furnace at 500°.

Use 100 ml of each distillate for colour development and measure the absorbance against a reference standard prepared as previously described. The standard curve is checked whenever a fresh suspension of calcium hydroxide is prepared.

Notes on Method

The two compounds most frequently used as a fixative for ashing samples for fluoride determination are magnesium acetate and calcium hydroxide. Although Crutchfield[14] and others have

* From Will Corp., Baltimore, Md., to hold thermometer.

found magnesium acetate very satisfactory as an ashing agent, fluoride samples dried in the presence of magnesium acetate showed some spattering when placed in a hot muffle furnace, causing recovery data to be inconsistent. However, good recoveries were observed if the dried samples were placed in a cool muffle furnace and heated gradually to the desired temperature. This latter procedure is time consuming when a large number of samples must be analysed. Although calcium hydroxide must be slaked to obtain a uniform suspension, it proved more satisfactory as a fixative than magnesium acetate in our work.

As shown previously, various investigators had used different temperatures for the ashing of bone samples prior to the distillation procedure. Table I shows the recovery of fluoride standards ashed in the presence of calcium hydroxide at various temperatures.

Table I. Effect of ashing temperature on fluoride recovery

Fluoride, μg	Temperature, °C	Time, h	Fluoride recovered, μg	% Recovery	Average % recovery
150	500	6	140	93	
150	500	6	146	97	
150	500	6	144	96	
150	500	6	146	97	96
150	600	6	144	96	
150	600	6	131	87	
150	600	6	144	96	
150	600	6	137	91	93
150	700	6	38	25	
150	700	6	56	37	
150	700	6	29	19	
150	700	6	49	33	29

Bumsted and Wells[8] studied the effect of the development time on colour intensity and found that there was a rapid change in colour intensity in the first 90 min. They recommended a development time of 2 h. Since the distillates collected by the present procedure contained between 80 and 120 μg, which is in the higher range, a longer bleaching period of 3 h was chosen.

The same authors recommended that a standard curve be run concurrently with each determination. In the present method it has been found necessary to run a standard curve only when a new supply of slaked calcium hydroxide suspension is prepared.

In agreement with Adams *et al.*,[15] the recovery of known amounts of fluoride is very much improved by using a standard curve which is run through the entire procedure. In this manner any loss of fluoride during ashing or distillation is inherent in the standard curve.

Fig. 1 compares the standard curves obtained by analysis of the distillate and by analysis of fluoride standards in water solution.

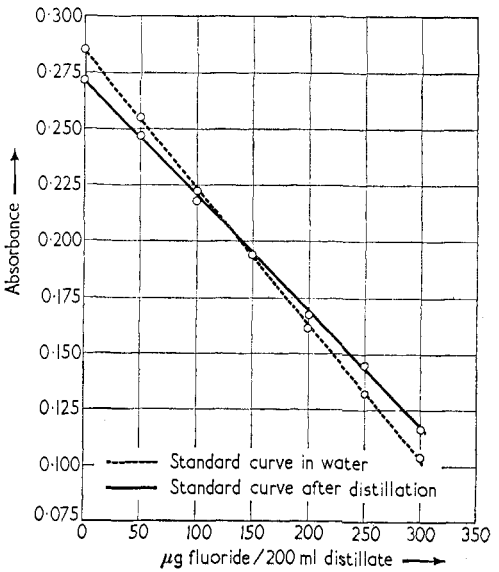

Fig. 1. Standard fluoride curves.

Recovery data were obtained on bone meal that had passed an 80-mesh screen. Fluoride standards, varying in concentration from 25–100 μg, were added to weighed quantities of sieved bone meal, and the total fluoride was determined by the standard

procedure. The recovery of the added fluorine ranged from 96 to 103 per cent (Table II).

Table II. Recovery of fluoride added to 0·2 gram of bone meal (passed through an 80-mesh screen)

Fluoride added, μg	Total fluoride recovered, μg	% Fluoride recovered
0	110	
0	108	
0	104	
25	133[a]	101
25	131	99
25	133	101
50	150[b]	96
50	152	97
50	158	101
100	205[c]	99
100	214	103
100	210	101

Theoretical fluoride in sample: [a] 132 μg; [b] 157 μg; [c] 207 μg.

Table III. Duplicate fluoride analyses on sections of dog femurs

Dog no.	Section of femur	Wt. of bone sample (mg)	μg of F^- per gm of bone	% Difference
1	Diaphysis	291·7	720	
		291·0	722	0·3
2	Diaphysis	208·0	720	
		210·4	697	3·3
3	Diaphysis	209·6	656	
		160·8	735	3·3
1	Epiphysis	183·4	1076	
		205·4	1071	0·5
2	Epiphysis	183·1	1227	
		110·7	1268	3·3
3	Epiphysis	196·8	1149	
		176·6	1138	1·0

Utilizing the present procedure, the fluoride content of dog femurs varied in different sections of the femur by as much as 40 per cent, as shown in Table III.

Animal Studies with 10-[3-(1-Methyl-4-piperazinyl)-Propyl]-2-Trifluoromethyl Phenothiazine Dihydrochloride (SKF No. 5019-A_2)

In the first study 60 adult albino rats, 30 males and 30 females, were divided into four groups, two control and two test groups, each group containing 15 rats. The test male and female rats were administered SKF No. 5019-A_2 by stomach tube at a dosage level of 1·0 mg/kg day for a period of 26 weeks. The rats were maintained on a basic laboratory diet and allowed tap water *ad libitum*. After the animals were sacrificed, the femurs were removed and a portion of each femur was analysed for fluoride content.

A second study was run using more closely controlled conditions, i.e. the animals were of a uniform size at the start of the experiment, the amount of fluoride ingested in the food was known and demineralized drinking water was allowed *ad libitum*. The test male and female rats were administered SKF No. 5019-A_2 by stomach tube at a dosage level of 10 mg/kg day for 75 days. After the animals were sacrificed, the femurs were removed and both femurs from each animal were analysed for fluoride content.

Discussion

In the first animal study only one femur from each rat was analysed for fluoride content. The bone was splintered, ground, and a portion of the ground bone was analysed. The intake of fluoride ion in the food and drinking water of the control and test animals was not known. Table IV shows the fluoride concentration obtained on the femurs of the rats used in this study. It is apparent that whether one considers each sex separately or averages them together, the average fluoride content of the test groups is essentially the same as that of the controls.

In the second study young male and female rats were maintained for 75 days on de-ionized water and a diet containing 4 ppm of fluoride ion. After the rats were sacrificed, both femurs in their

Table IV. First rat study—test animals received 1 mg of SKF 5019-A_2 per kg per day. Fluoride content of rat femurs: micrograms of F^- per gram of femur

Rat no.	Males		Females	
	Control	Test	Control	Test
1	608	526	752	896
2	698	619	826	862
3	798	876	923	826
4	684	788	857	797
5	881	695	826	845
6	681	794	725	768
7	656	703	922	879
8	743	645	783	847
9	576	750	752	874
10	709	597	855	750
11	758	676	1000	852
12	691	705	726	909
13	666	680	850	847
14	895	635	929	938
15	—	730	931	827
Average	717	695 (−3·1%)	844	848 (+0·3%)

entirety were analysed for fluoride content. The results obtained on the male and female rats are listed in Tables V and VI respectively. Once again it is apparent that SKF No. 5019-A_2, based on the analysis of μg of F/g of femur, had no effect on increasing the fluoride content of the test animals over that of the controls.

In this study, in some rats, both male and female, the micrograms of fluoride per gram of femur were the same for the two femurs, while in others there was a considerable difference between the femurs. The standard deviation of a single femur in the control groups, based on the differences between the two femurs for each animal, is approximately 9 for males and 15 for females. This means that 95 per cent of the time the two femurs for a given animal should differ by no more than 36 for males and 60 for females. In all cases the differences in the fluoride content between femurs in the test animals are within these ranges.

At the time of sacrifice the control rats from the first study had a larger concentration of fluoride ion in the femur than those from the second study. In the first study the animals, males and females, were maintained on tap water and a standard laboratory diet. In the second study the animals were maintained on de-

Table V. Second rat study (males)—test animals received 10 mg of SKF No. 5019-A_2 per kg per day

Rat no.	Control group		Test group	
	Wt. of femur (mg)	μg of F^- per gm of femur	Wt. of femur (mg)	μg of F^- per gm of femur
1	664·1	148	565·4	166
	667·2	123	570·7	144
2	641·3	125	391·8	130
	709·1	138	423·2	137
3	778·9	144	672·2	134
	750·0	141	668·4	114
4	655·2	108	558·7	122
	638·6	111	666·9	135
5	631·4	147	656·2	125
	650·3	155	664·6	117
6	796·5	148	688·5	164
	827·3	160	684·4	158
7	847·7	132	811·8	139
	882·1	141	810·5	144
8	816·5	167	713·4	121
	779·2	159	700·3	114
9	578·9	157	505·7	162
	624·7	143	520·5	135
10	785·4	158	553·4	148
	801·1	142	556·9	151
Average		142		138 (−2·8%)

mineralized water and a standard diet. On the basis of the first study *versus* the second study, the average value for micrograms of fluoride per gram of femur in the males was 717 *vs* 142, and in the females 844 *vs* 174. Normal growth (weight gain) charts were obtained in both studies. These data confirm what is already

known, i.e. increasing concentrations of inorganic fluoride in the diet cause an increased uptake of fluoride by bone.

The experimental data from both studies show that there is no increase in the fluoride content of the femurs of the test animals over those of the controls. Hence it is felt that this is suggestive

Table VI. Second rat study (females)—test animals received 10 mg of SKF 5019-A_2 per kg per day

Rat no.	Control group		Test group	
	Wt. of femur (mg)	μg of F^- per gm of femur	Wt. of femur (mg)	μg of F^- per gm of femur
1	527·7	176	470·3	128
	503·8	201	466·8	124
2	570·5	186	462·8	147
	534·0	170	429·4	182
3	499·3	176	473·8	220
	491·9	189	523·6	191
4	518·9	154	507·5	197
	435·9	138	411·7	199
5	484·6	167	419·9	200
	506·4	164	502·3	179
6	596·8	142	410·3	215
	593·1	120	446·5	229
7	438·8	162	462·9	—
	481·1	168	473·3	165
8	542·2	210	472·1	153
	551·0	187	435·6	147
9	461·3	184	494·1	126
	460·4	213	484·4	140
10	469·0	203	427·7	229
	392·1	163	437·6	229
Average		174		178 (+2·3%)

evidence that the trifluoromethyl group attached to a phenothiazine nucleus is stable in an *in vivo* system.

Summary. In order to ascertain the *in vivo* stability of the trifluoromethyl group attached to a phenothiazine nucleus, 10-[3-(1-methyl-4-piperazinyl)-propyl]-2-trifluoromethyl phenothiazine dihydrochloride (SKF

No. 5019-A_2) was administered to two groups of rats at dosage levels of 1 mg/kg day and 10 mg/kg day for 182 and 75 days, respectively. The femurs of these animals were analysed for fluoride content, as it was postulated that instability of the trifluoromethyl group would cause an increase in fluoride content of bone. The results of the experiments indicate that the trifluoromethyl group is stable in an *in vivo* system.

Also a spectrophotometric method is described for the determination of the fluoride in bone. Fixatives, temperature, colour development and preparation of the standard curve are discussed, and recovery data are presented. Analysis of different sections of dog femurs showed as much as 40 per cent variation in the same animal.

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