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Fluorescence of Boron Complexes. VI.[1] Fluorometric Determination of Salicylic Acids[2]

Toshio Shibazaki, Tokiko Nishimura, Machiko Hara, and Tadashi Iijima

National Institute of Hygienic Sciences[3]

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The fluorescence reaction of salicylic acid and its derivatives with boric acid was successfully applied to their micro-analyses.

The recommened procedures were as follows: Seven milliliters of the glacial acetic acid solution of a sample of salicylic acids (5×10^{-6}—5×10^{-8} M) was mixed with 1.0 ml of sulfuric acid-glacial acetic acid solution with concentration represented in Table II, 1.0 ml of acetic anhydride, and 1.0 ml of boric acid-glacial acetic acid solution, stoppered and allowed to keep at 60° for 10 min. Then the fluorescence intensity of the solution was measured at the excitation and fluorescence maxima of each of the samples shown in Table II. In the case of 4-hydroxysalicylic acid and 5-aminosalicylic acid, 8.0 ml of each solution was mixed with 1.0 ml of 5% boric acid–acetic anhydried solution, and the mixture was kept at 60° for 10 min. Then 1.0 ml of 0.01 N sulfuric acid solution in-glacial acetic acid was added to the mixture.

The coefficients of variation for these four compounds (1×10^{-6} M) were 0.8—1.0%. Among diverse compounds, quinie ethylcarbonate disturbed the determination.

This fluorometry was applied to the determination of salicylid acid in fermented liquor, blood plasma and urine, and the analytical results agreed well with those obtained by the colorimetric method.

Keywords——salicylic acids; fluorometry; boron complex; sake; urinary excretion; blood plasma level

In our previous paper, we reported the relationship between the fluorescence intensity of boron complexes of salicylic acid esters and the structures,[1] and also established fluorometric determination of dehydroacetic acid, salicylic acid esters, salicylaldehydes, and *o*-hydroxyphenylketons with boric acid.[4–6] In the present paper, the fluorometric determination of nucleophilic substituted salicylic acids was established by the application of this fluorescence reaction. The method was further developed for measurement of salicylic acid (SA) in sake, total SA in human urine after oral administration of aspirin, and unchanged SA in rabbit plasma after intravenous injection of sodium salicylate solution.

Most of the 16 kinds of salicylic acids investigated (Table I) showed larger reactivity and intense fluorescence, as the ratio of the glacial acetic acid (glacial AcOH) and amount of boric acid (H_3BO_3) in glacial AcOH–acetic anhydride solution increased. The solution sometimes became cloudy under the presence of trace amounts of moisture. Addition of small amounts of sulfuric acid, however, made it possible to remove the influence of moisture and brought about good results with a relatively low concentration of H_3BO_3. And then the excitation maxima, the fluorescence maxima, and fluorescence intensities of the solutions of many salicylic acids did not change appreciably, except 6-hydroxysalicylic acid, 3-hydroxysalicylic acid, and 5-aminosalicylic acid. The excitation maximum of the fluorescence solution of 6-hydroxysalicylic acid was shifted from 350 nm to 334 nm and the fluorescence maximum

1) Part V: T. Shibazaki and S. Yoshioka, *Yakugaku Zasshi*, **94**, 1585 (1974).
2) Presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, July 1970.
3) Location: *Kamiyoga, Setagaya-ku, Tokyo 158, Japan.*
4) T. Shibazaki, *Yakugaku Zasshi*, **88**, 601 (1968).
5) T. Shibazaki, *Yakugaku Zasshi*, **88**, 1393 (1968).
6) T. Shibazaki, *Yakugaku Zasshi*, **90**, 413 (1970).

from 403 nm to 412 nm. And the intensity reduced approximately by half, but became stable. The fluorescence intensity of the solution of 3-hydroxysalicylic acid increased and that of 5-aminosalicylic acid decreased. 3-Aminosalicylic acid did not show measurable fluorescence even by the addition of sulfuric acid.

The continuous variation method suggested that the fluorescence reaction was resulted from the formation of the 1:1 complex (chelate) of SA and boron, as reported previously.[1] The excitation and fluorescence maxima of SA by this boron complex method were found at longer wavelength than those reported by Chirigos and Udenfriend,[7] respectively and the fluorescence intensity was about 3 times stronger (uncorr.), and was suited for microanalyses.

Table I. Absorption and Fluorescence Characteristics of Boron Complexes of Salicylic Acids[a]

Compound No.	Substituent R	mp (°C)	Boron complex: Absorption λ maximum (nm)	Boron complex: Concentration: 2×10^{-6} M: Excitation λ max[b] (nm)	Boron complex: Concentration: 2×10^{-6} M: Emission λ max[c] (nm)	R.F.I.[d]
I	H	160	334	336	407	460
II	3-OH	207	335	335	425	240
III	4-OH	225	316	316	385	220
IV	5-OH	203	341	340	426	420
V	6-OH	164	335	334	412	280
	6-OH[e]		348	350	403	580
VI	3-NH_2	232	330—345	—	—	none
VII	4-NH_2	153	295	293	391	690
			333sh[f]	332sh	391	270
VIII	5-NH_2	280	340—355	340—355	460	90
IX	3-CH_3	166	338	338	426	330
X	4-CH_3	178	334	334	405	380
XI	5-CH_3	154	350	350	425	420
XII	5-Br, 3-CH_3	235	349	348	429	50
XIII	3-ø	208	347	348	452	260
XIV	4-Cl	209	330	329	363	70
XV	5-COOH	302	327	326	394	200
XVI	5-SO_3H	118	310—326	313—326	498	190
	Quinine sulfate[g]		347	347	452	1000

a) The measurement conditions are the same as in Table II.
b, *c*) The excitation and emission maxima are corrected values.
d) Relative fluorescence intensity.
R.F.I. of salicylic acids were compared to the same molar solution of quinine sulfate in 0.1 N H_2SO_4.
e) Same as *f*) in Table II
f) Shoulder.
g) $(C_{20}H_{24}N_2O_2)_2\cdot H_2SO_4\cdot 2H_2O$, mw 782.97.

Experimental

Apparatus and Conditions——Fluorescence was measured with Hitachi recording fluorescence spectrophotometer, Model MPF-2A, used under the following conditions: Applied dynode voltage, 750 V; slit width, 6 nm on excitation side, 6 nm on fluorescence side; sensitivity, 0.1 to 10.

Correction for excitation and fluorescence spectra were the same as reported previously.[6]

Hitachi recording spectrophotometer, Model EPS-3T, was used for measurement of absorption spectra.

7) M. Chirigos and S. Udenfriend, *J. Lab. Clin. Med.*, **54**, 769 (1959).

Reagents——One per cent boric acid–glacial AcOH solution (1% H_3BO_3–AcOH solution): One gram of finely powdered H_3BO_3 was dissolved with shaking in 100 ml of glacial AcOH. Sulfuric acid–glacial acetic acid solution (H_2SO_4–AcOH solution): One normal sulfuric acid in glacial acetic acid was prepared by slowly adding 5.0 g of sulfuric acid dropwise to 100 ml of glacial AcOH with shaking in a water bath of 16°. To this solution, glacial AcOH was added at the time of use to prepare 0.1 N, 0.03 N, 0.02 N, 0.01 N, and 0.001 N H_2SO_4–AcOH solutions. Quinine sulfate standard stock solution and quinine sulfate standard solution: The standard stock solution was prepared by dissolving 78.3 mg of quinine sulfate [$(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$: 782.95] in 0.1 N sulfuric acid and making up to 1000 ml (10^{-4} M). As the standard solution, 2×10^{-6} M, 2×10^{-7} M, and 5×10^{-8} M solution were prepared by adding 0.1 N sulfuric acid to this stock solution at the time of use. Chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) were special reagent grade of Japanese Industrial Standard and distilled at the time of use. Acetic anhydride (Ac_2O), glacial acetic acid (glacial AcOH), boric acid–acetic anhydride solution (H_3BO_3–Ac_2O solution) were the same as those reported previously.[6] The other reagent solutions used were those conforming to the Japanese Pharmacopoeia IX.

Materials——Salicylic acids were obtained commercially. The salicylic acids of which melting points did not agree with literature values were recrystallized. Each melting point (corr.), containing decomposition points, was listed in Table I.

Fluorescence Determination——As shown in Table II, 7.0 ml of the glacial AcOH solution of a material (5×10^{-6}—1×10^{-7} M) in glass stoppered tube is mixed with 1.0 ml of Ac_2O, 1.0 ml of H_2SO_4–AcOH solution of the concentration given in Table II, and 1.0 ml of 1% H_3BO_3–AcOH solution, stoppered, and allowed to keep at 60° for 10 min. After cooling, the fluorescence intensity of the solution transferred into a stoppered fluorescence cell of 1×1 cm optical path length is measured at the excitation and fluorescence maxima of the compound shown in Table II. At the same time, the reagent blank value obtained in the same manner is subtracted from the value. On the other hand, for compound III (4-hydroxysalicylic acid) and VIII (5-aminosalicylic acid) of which fluorescence is unstable under the above condition, 8.0 ml of glacial AcOH solution of the compound is mixed with 1.0 ml of 5% H_3BO_3–Ac_2O solution, stoppered, and kept at 60° for 10 min. After cooling, the solution is mixed with 1.0 ml of 0.01 N H_2SO_4–AcOH solution, and the fluorescence intensity of the mixture is measured.

Table II. Conditions for the Fluorometry of Salicylic Acids[a]

Compound (No.)	Concentration of H_2SO_4–AcOH solution (N)	Excitation λ max[b] (nm)	Emission λ max[c] (nm)	Suitable concentration range for fluorometry[d] (M)
I	0.03	339	404	2×10^{-8}—5×10^{-6}
II	0.03	340	420	5×10^{-8}—5×10^{-6}
III	0.01[e]	322	384	8×10^{-8}—5×10^{-6}
IV	0.03	346	424	2×10^{-8}—5×10^{-6}
V	1.0	366	408	5×10^{-8}—5×10^{-6}
V	0[f]	350	400	2×10^{-8}—5×10^{-6}
VII	0.03	335	391	5×10^{-8}—5×10^{-6}
VIII	0.01[e]	354	453	1×10^{-7}—1×10^{-5}
IX	0.03	344	423	2×10^{-8}—1×10^{-6}
X	0.03	338	403	2×10^{-8}—5×10^{-6}
XI	0.03	350	422	2×10^{-8}—5×10^{-6}
XII	0.03	351	426	2×10^{-7}—5×10^{-6}
XIII	0.03	353	448	5×10^{-8}—5×10^{-6}
XIV	0.03	332	392	2×10^{-7}—5×10^{-6}
XV	0.03	331	394	1×10^{-7}—1×10^{-5}
XVI	0.03	332	398	1×10^{-7}—1×10^{-5}
Quinine sulfate in 0.1 N H_2SO_4		351	446	1×10^{-8}—5×10^{-6}

a) To make a fluorescence solution, 7 ml of glacial AcOH solution of compound is mixed with 1 ml of Ac_2OH, 1 ml of H_2SO_4–AcOH solution of a concentration show in this table and 1 ml of 1% H_3BO_3–AcOH solution except compound III, VIII and *f*) of V. The mixture is then kept at 60° for 10 min.

b, *c*) The exciation and emission maxima are not corrected.

d) Calibration curves are indicated in strait lines in these concentration ranges.

e) To make the fluorescence solution, 8 ml of glacial AcOH solution of the compound is mixed with 1 ml of 5% H_3BO_3–Ac_2O solution. The mixture is kept at 60° for 10 min and cooled. Then 1 ml of 0.01 N H_2SO_4–AcOH solution is added into the mixture.

f) To make the fluorescence solution, 9.0 ml of glacial AcOH solution of V is mixed with 1 ml of 5% H_3BO_3–Ac_2O solution, the mixture is then kept at 60° for 10 min.

Results and Discussion

Conditions for Fluorescence

In order to obtain a stable and strong fluorescence intensity of salicylic acids, various conditions were examined. In the case of estimation of fluorescence intensity, the final concentration of each of these was 2×10^{-6} M and the excitation and fluorescence wavelengths used followed Table II.

Solvents——Relationship between the fluorescence intensity and a mixing ratio of glacial AcOH and Ac_2O in the measurement solution was investigated in the range of 10: 0 to 4: 6. In this experiment, the final concentration of H_3BO_3 was 0.1% and that of H_2SO_4 was 0.003 N. After heating at 60° for 10 min and cooling to room temperature, the fluorescence intensity of the solution tended to increase in proportion to the amount of glacial AcOH except compound III but still 2% of Ac_2O was required as the minimum. The fluorescence intensity of III became stronger at the mixing ratio of 4: 6 but unstable, and reduced approximately by two thirds in the mixing ratio of the range, 9: 1 to 9.5: 0.5.

Sulfuric Acid Concentration——Since it was found that the addition of a trace of H_2SO_4 prevented the cloudiness of the measurement solutions, the suitable concentration of H_2SO_4 for each salicylic acids in the solutions was examined. Each of compound I, II, IV, VII and IX—XVI in Table I indicated the similar fluorescence intensity in the range of 0.001 to 0.01 N H_2SO_4 in the solution as shown in Table III. The excitation maximum of 350 nm and fluorescence maximum of 403 nm of the measurement solution of compound V free from H_2SO_4 varied with increasing concentration of H_2SO_4, and reached nearly constant at the concentration higher than 0.1 N, while the wavelengths had shifted to 334 nm and 412 nm, respectively. The fluorescence intensity then reduced approximately by half (Table I). The fluorescence intensity of the solutions of compound III and VIII decreased with increasing concentration of H_2SO_4 and became unstable.

Table III. Effect of Sulfuric Acid Concentration in Measurement Solution on Fluorescence Intensity (%)

Compound[a] No.	Concentration of H_2SO_4 (N)[b]			
	0.0001	0.001	0.01	0.1
I	42	45	46	43 (%)
II	33	45	45	44
III	37	35	25	21
IV	34	44	46	46
V	36	38	59	63
VII	40	45	43	41
VIII	40	42	28	10
IX	41	46	44	41
X	43	46	46	44
XI	40	41	42	40
XII	35	38	38	37
XIII	35	39	38	37
XIV	34	36	36	33
XV	35	40	40	40
XVI	32	34	36	33

a) Concentration of compound: 2×10^{-6} M.
b) Concentration of H_2SO_4 in a medium consisted of 8 volume of glacial AcOH, 1 volume of Ac_2O and 1 volume of 1% H_3BO_3-AcOH solution.
Reaction: 60°, 10 min.

Boric Acid Concentration——The concentration of H_3BO_3 in measurement solutions was concluded to be 0.1%, because many of salicylic acids showed the similar fluorescence intensity

in the range of 0.1—0.5% as represented in Table IV. Since stable fluorescence was not obtained in compound III and VIII as descrived above, the following examination was carried out. To 3.0 ml of the glacial AcOH solution of each, 1.0 ml of Ac_2O and 0.1 to 5 ml of 1% H_3BO_3–AcOH solution were added, diluted to 9.0 ml with glacial AcOH, and shaken. After heating and cooling, 1.0 ml of 0.01 N H_2SO_4–AcOH solution was added to the above mixture for preventing the cloudiness during the measurement. Consequently stable fluorescence was found in the concentration of 0.5% H_3BO_3. From the above results, as shown in Table II,

Table IV. Effect of Boric Acid Concentration in Measurement Solution on Fluorescence Intensity (%)

Compound[a]	Concentration of H_2SO_4 (N)	Concentration of H_3BO_3 (%)[b]		
		0.01	0.1	0.5
I	0.003	59	64	63
II	0.003	31	33	34
III	0.001[c]	12	66	68
IV	0.003	64	69	68
V	0.1	30	32	33
VII	0.003	31	33	34
VIII	0.001[c]	28	43	45
IX	0.003	42	46	46
X	0.003	50	48	48
XI	0.003	50	46	45
XII	0.003	62	63	64
XIII	0.003	35	40	42
XIV	0.003	33	35	35
XV	0.003	48	50	52
XVI	0.003	40	43	44

a) Concentration of compound: 2×10^{-6} M.
b) Concentration of H_3BO_3 in a measurement solution consisted of 9 volume of glacial AcOH, 1 volume of Ac_2O and concentration of H_2SO_4 shown in this Table.
c) The condition is the same as *e*) in Table II except concentration of H_3BO_3. Reaction: 60°, 10 min.

Table V. Effect of Heating Time of Measurement Solution on Fluorescence Intensity (%)

Compound[a] No.	Heating time (min)			
	5	10	20	40
I	49	47	46	46 (%)
II	37	36	37	37
III	50	62	63	65
IV	36	35	35	35
V	42	43	45	45
VII	45	52	53	52
VIII	39	42	44	44
IX	41	37	37	37
X	54	52	53	52
XI	41	40	40	40
XII	39	37	37	37
XIII	34	34	34	34
XIV	40	40	40	39
XV	39	38	38	38
XVI	40	38	39	38

a) Concentration of compound: 2×10^{-6} M.
Conditions are the same as Table II except heating time.

1.0 ml of 5% H_3BO_3–Ac_2O solution was added to 8.0 ml of glacial AcOH solution of each, heated, and cooled. To the mixture, 1.0 ml of 0.01 N H_2SO_4–AcOH solution was added.

Heating Time and Stability

The measurement solutions prepared under the resulting conditions represented in Table II were kept at 60° for 5 to 40 min and the fluorescence intensities of the solutions were measured at room temperature. The intensities of each of salicylic acids were stable on heating for 10 min and did not change even heating for 40 min (Table V).

Calibration Curves and Precision

The calibration curves were made according to the fluorometric determination based on the above results. Appropriate linearity was observed between the concentration and the fluorescence intensity for each of salicylic acids in the concentration range as shown in Table II.

Repeated runs were carried out 7 times according to the fluorometric determination for compound I, III, VIII, and XVI. Reproducibility in the final concentration of 1×10^{-6} M of the compounds was as follows: Compound I; σ (standard deviation)$=0.3_3$, cv(coefficient of variation)$=0.6_3\%$. III; $\sigma=0.4_2$, $cv=0.8_1\%$. VIII; $\sigma=0.5_1$, $cv=1.0_4\%$. XVI; $\sigma=0.4_0$, $cv=0.7_9\%$.

Effect of Foreign Organic Materials

To measurement solution of 2×10^{-6} M SA, each of other compounds was previously added and fluorescence intensity of the solution was measured. The effect of foreign com-

Table VI. Effect of Foreign Oragnic Materials on Determination of Salicylic Acid

Compound	Amount added (μg/ml)	Recovery of salicylic acid[a] (%)
Aminopyrine	1.39	100
Aminophyline	2.74	101
Antipyrine	1.11	99
Acetanilide	0.83	101
o-Etyoxybenzamide	0.98	107
		101[b]
4-Isopropylantipyrine	1.40	101
Acetaminophen	0.91	101
Phenacetin	1.06	99
Diphenhydramine HCl	1.54	99
Chlorpheniramine maleate	2.35	99
Methylephedrine HCl	1.31	101
Sulpyrine	2.10	102
Quinine ethylcarbonate	2.34	121
Barbital	1.11	100
Caffeine anhydride	1.14	101
p-Hydrobenzoic acid	0.85	99
Ethylparaben	1.07	100
Benzoic acid	0.75	100
Thiamine nitrate	1.98	101
Ascorbic acid	3.63	100
Taurine	2.53	100
Lactose	7.20	100
Citric acid	4.20	100
Ethanol	46.0	100
Ethylacetate	54.6	100

a) Concentration of salicylic acid in a final solution: 0.276 μg/ml (2×10^{-6} M). Excitation 340 nm, emission 405 nm.
b) Excitation 355 nm, emission 415 nm.

pounds was hardly observed except ethoxybenzamide and quinine ethylcarbonate as listed in Table VI. Ethoxybenzamide did not affect by using the excitation wavelength of 350 nm and fluorescence wavelength of 410 nm.

Composition of Complexes

The ultraviolet absorption maximum of the fluorescence solution for each of salicylic acids was observed at a longer wavelength than that of the 98% glacial AcOH solution of the corresponding one. The excitation maxima of these fluorescence solutions agreed with the corresponding absorption maxima (Table I). The formation of boron complexes was estimated from the above observation. The bonding ratio of SA and boron was calculated by the continuous variation method and was found to be 1:1 as shown in Fig. 1. The results suggested that these complexes were the same type structure of chelate as in the case of salicylic acid esters reported previously.[1)]

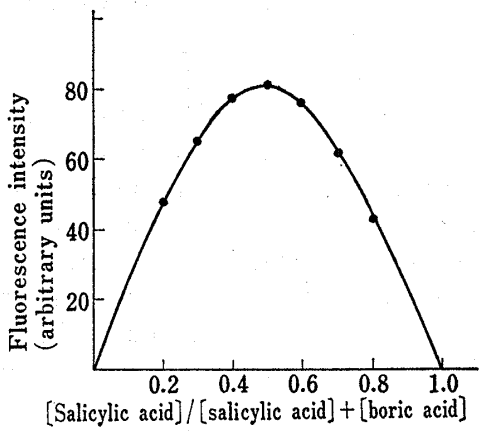

Fig. 1. Continuous Variation Method
[Salicylic acid]+[boric acid]=5×10^{-5} M.
Excitation: 370 nm, emission: 405 nm.

Application to Determination of SA in Sake and Body Fluids

We attempted the application of this fluorometry to the determination of SA in sake, blood, and human urine. The resulting values agreed with those obtained by the application of the colorimetry[8,9)] with ferric salt for the same sample, and confirmed the high reliability of the microassay by using this fluorometry (Fig. 2, 3). The absorbance of 0.5 mM concentration of SA in ferric nitrate solution (pH 2.4) was about 0.84 in a 1-cm path length cell at 530 nm and was influenced with changes in pH value.

Determination of SA in Sake and Wine——The amount of SA in sake was regulated to less than 0.025% w/v.[10)] In the fluorometry 1.0 ml of glacial AcOH diluent of sake (1→500) was used as follows for the fluorescence reaction, while in the colorimetry by the modification of procedure A of Levy and Procknal[9)] the SA in 2.0 ml of sake was extracted with 10 ml of CCl_4 after HCl-acidizing and a 6.0 ml aliquot of the organic phase was required to the determination of SA.

Fluorometry: Forty microliters of sake[11)] and 15% v/v ethanol solution of 0.0250% SA (standard solution) is mixed with 20.0 ml of glacial AcOH respectively. To 1.0 ml of each solution, 9.0 ml of a mixed solution (0.1% BSA solution) of 0.03 N H_2SO_4–AcOH solution, glacial AcOH, 1% H_3BO_3–AcOH solution, and Ac_2O (1:7.5:1:0.5) is added, kept at 60° for 10 min, and cooled to room temperature. Each fluorescence intensity of the mixtures is measured at excitation wavelength of 340 nm and fluorescence wavelength of 405 nm and a blank test value is subtracted from the resulting value. The amount of SA in sake is obtained by comparing with the intensity of the standard measurement solution.

The amount of SA in wine determined by the above fluorometry was about 5% lower than that of the added. The determination of SA was therefore carried out as follows: 0.50 ml of the wine sample, 0.5 ml of 0.5 N HCl and 10.0 ml of CCl_4 were placed in a stoppered test tube. After shaking, 0.50 ml of the organic phase was mixed with 0.1% BSA solution as described above.

8) W. Chiou and I. Onyenmelukwe, *J. Pharm. Sci.*, **63**, 630 (1974).
9) G. Levy and J.A. Procknal, *J. Pharm. Sci.*, **57**, 1330 (1968).
10) Adding of SA into sake had been regulated under Article 6, 7 and Table II of the Food Sanitation Act of Japan, but it was prohibited to use by Notification No. 238 on July 25, 1975. On the other hand, it still is adding in wine of France made.
11) Bought on July 1968 and determined on March 1969.

Results: The amounts of SA in two kinds of sakes and a kind of wine were determined by the above fluorometry and the colorimetry. The results were given in Table VII.

Determination of SA in Biological Samples

In order to measure a small amount of SA in body fluids, it would be necessary to separate the contaminants. For this purpose, SA was extracted with $CHCl_3$ by Chiou and Onyenmelukwe,[8] or with CCl_4 by Levy and Procknal,[9] reextracted into a ferric nitrate solution, and determined colorimetrically. We considered that a given amount of $CHCl_3$ extract, or CCl_4 one, of SA was mixed with BSA solution and determined fluorometrically. The effect of these solvents for extraction on the fluorescence intensity was then examined. Gentisic acid, metabolite of SA, was not extracted into these solvents. Each of the organic phases was able to treat as the fluorometric determination described above. Consequently, it was found that the amount of SA in human urine or rabbit plasma was needed more than about 0.5 or 3 μg per ml for the following fluorometry, whereas, for the colorimetry, about 20 or 60 μg, respectively.

Determination of Total SA in Human Urine——Urine after oral administration of one aspirin enteric-coated tablet (containing 0.25 g/tablet) in man subject was collected periodically and used as urine samples.

Fluorometry: Total SA(SA and salicyluric acis and salicylic glucuronides) in urine is determined after complete acid hydrolysis of these metabolites to SA. According to the colorimetry for the total SA in human urine by Chiou and Onyenmelukwe,[8] the SA in 3.0 ml of urine sample treated with HCl is extracted into 6.0 ml of $CHCl_3$. The organic layer is placed in a stoppered centrifuge tube and centrifuged (2000 rpm, for 5 min) to remove majority of moisture. The $CHCl_3$ layer is collected, taking care not to mix the water droplets, and to 0.5 ml of it, 5.0 ml of 0.1% BSA solution is added as described above. 3.0 ml of the urine, which was collected before administration of aspirin in the same manner as the urine sample and the solution obtained by adding 15.0 μg (100 μl) of sodium salicylate to 3.0 ml of the above urine, is used for preparation of the blank test solution and the comparative standard fluorescence solution.

Results: The results of determination obtained by the fluorometry and the colorimetry reported by Chiou and Onyenmelukwe[8] were illustrated in Fig. 2.

Table VII. Comparison of Analytical Results for Salicylic Acid in Sake and Wine by the Present Method and the Colorimetry

Sample			Fluorometry (%)	Colorimetry (%)
Sake[a]	A		0.024	0.024
			0.024	0.023
			0.023	
		Mean value	0.024	0.023
	B		0.022	0.023
			0.021	0.022
			0.022	
		Mean value	0.022	0.022
Wine[b]			0.025	0.025
			0.025	0.026
			0.025	0.025
		Mean value	0.025	0.025

a) These were bought on July 1968 and determined on March 1969.
b) Two hundred fifty mg of salicylic acid were added in 1000 ml of wine (JP IX).

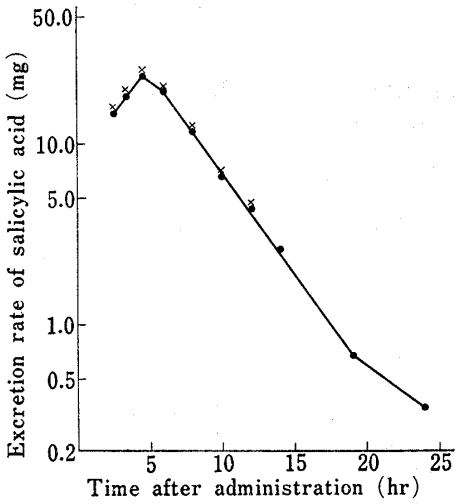

Fig. 2. Urinary Excretion of Salicylic Acid after Oral Administration of Aspirin (250 mg) Enteric-coated Tablet in Man

●: by fluorometry, ×: by colorimetry.

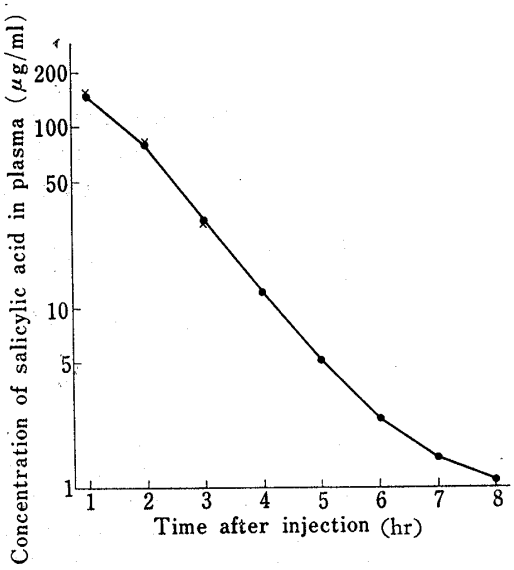

Fig. 3. Blood Plasma Levels of Salicylic Acid after Intravenous Injection of Sodium Salicylate (300 mg) into Rabbit

●: by fluorometry, ×: by colorimetry.

Determination of SA in Rabbit Plasma——Blood was collected periodically after intravenous injection of 2.0 ml (0.30 g) of sodium salicylate solution into rabbit.

Fluorometry: For the extraction of SA from the blood, the extract technique of SA from the urine by Levy and Procknal[9] is referred to. The blood (0.8 ml) is collected with a 1.00 ml injector previously containing 0.2 ml of diagnostic citrate (JP IX), transferred to a small stoppered centrifuge tube, mixed gently, and after allowing the mixture to stand, centrifuged to collect the plasma. To 0.50 ml of the supernatant solution, 0.5 ml of 6 N HCl and 8.0 ml of CCl_4 are added, and the mixture is shaken carefully. The organic phase (T) is then transferred into a stoppered cantifuge tube and subsequently treated in the same manner as above human urine. The solution (B) obtained by treating the blood collected before intravenous injection of sodium salicylate in the same manner as the preparation of solution T is used for blank test. The solution (S) for preparation of the comparative standard solution is obtained by using an injector containing 50 μg of sodium salicylate and 0.20 ml of diagnostic citrate in the same as preparation of solution B.

Results: The results of determination obtained by the fluorometry and the colorimetry reported by Levy and Procknal[9] were shown in Fig. 3.

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