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Fluorescence of Boron Complexes. VII.[1] Fluorometric Determination of Salicylamide

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Fluorometric determination of salicylamide was studied. Salicylamide fluoresced strongly when excited in a mixed solution of 0.1% boric acid, 0.005 N sulfuric acid, and 5% acetic anhydride in glacial acetic acid. This fluorophore was a salicylamide–boron–acetic acid (1:1:2) complex. The fluorescene emission maximum occurred at 388 nm, and excitation maximum at 328 nm. The linear range in the calibration curve was 5—1000 ng/ml in the final solution, coefficient of variation for salicylamide (0.28 μg/ml) in the measurement ten times was 0.6%. This method was about five times more sensitive than the fluorometric method reported by Barr and Riegelman. The proposed method was successfully applied to the determination of salicylamide in urine samples, in which minimum concentration was *ca.* 30 ppb.

Keywords——salicylamide; fluorometry; urinary excretiom; boron-complex; boric acid

Recently, Fleckenstein and others[3] used the fluorometric method of Barr and Riegelman[4] for the determination of unchanged salicylamide (SAM) in human plasma. However, the fluorescence is somewhat week in this method and not so stable. In application of this method for the estimation of SAM in urine, the blank value determination is difficult and it was difficult to evalute the amount of SAM in urine less than about 0.5 ng/ml with a high accuracy.

SAM ingested by humans is mostly metabolized into salicylamide sulfate, salicylamide glucuronide, gentisamide glucuronide, and salicyluric acid, and the amount of unchanged salicylamide is said to be extremely small.[4,5] There seems to be no report on the measurement of unchanged salicylamide in urine with high reliability. Therefore, we attempted the application of the fluorescence reaction of salicylic acids with boric acid (H_3BO_3), reported in the preceding paper,[1] to the estimation of SAM. In order to measure the trace amount of SAM in urine with high precision, it would be necessary to fully examine the reagent blank and urine blank, so that detailed examinations were made on these points in the present work.

Salicylic acids show a stable fluorescence in a mixed solution of glacial acetic acid–acetic anhydride (9:1) containing 0.1% H_3BO_3, in the presence of a trace of sulfuric acid. It had been found that the intensity of this fluorescence is generally greater, the larger the ratio of glacial acetic acid (glacial AcOH) in the mixture.[1] On the other hand, SAM shows a stable fluorescence irrespective of the mixing ratio of glacial AcOH and acetic anhydride (Ac_2O), and its calibration curve is linear in the range of 1 μg to 5 ng/ml measuring solution. The intensity of this fluorescence is about 0.9 fold (uncorr.) of quinine sulfate (mol. wt. 782.95, 0.1 N sulfuric solution) in equimolar comparison. The intensity is about 5-fold (uncorr.) of

1) Part VI: T. Shibazaki, T. Nishimura, M. Hara, and T. Iijima, *Chem. Pharm. Bull.* (Tokyo), **26**, 1737 (1978).
2) Location: *Kamiyoga, Setagaya-ku, Tokyo 158, Japan.*
3) L.Fleckenstein, G.R. Mundy, R.A. Horovity, and J.M. Mazzullo, *Clin. Pharmacol. Ther.*, **19**, 451 (1976).
4) W.H. Barr and S. Riegelman, *J. Pharm. Sci.*, **59**, 154 (1970).
5) G. Levy and T. Matsuazwa, *J. Pharmacol. Exp. Ther.*, **156**, 285 (1976).

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that obtained by the method of Barr and Riegelman,[4] and is suitable for the measurement of a minute amount.

Experimental

Apparatus and Conditions——These were the same as reported in the preceding paper.[1] Measurement of fluorescence, and correction for excitation and fluorescence spectra followed the same manner as reported previously.[6]

Reagents and Materials——SAM (Mol. Wt. 137.14): Japanese Pharmacopoeia VIII commercial product was recrystallized from 80% (v/v) ethanol.

0.1% BSA solution: To 10 ml of 1% H_3BO_3–AcOH solution in stoppered flask, 80 ml of glacial AcOH, 5 ml of 0.1 N H_2SO_4–AcOH solution, and 5 ml of Ac_2O were added with shaking in a water bath of *ca.* 15°. This solution was prepared at the time of use.

Other reagents and reagent solutions used were the same as those described in the preceding paper.[11]

Fluorometric Determination of SAM——A mixture of 2.0 ml of glacial AcOH or dichloroethane (CH_2Cl-CH_2Cl) solution containing 5 μg to 30 ng of SAM amd 4.0 ml of 0.1% BSA solution is placed in a glass-stoppered test tube, stoppered, and allowed to stand at room temperature for more than 20 min. The solution is then transferred into a fluorometric cell and fluorescene intensity of the solution is measured at the excitation and fluorescene wavelength of 328 nm and 388 nm, respectively. A mixture of 4.0 ml of 0.1% BSA solution and 2.0 ml of glacial AcOH or dichloroethane is used as the reagent blank.

Application to Human Urine Sample——1) Extraction of SAM from Human Urine: In a stoppered centrifuge tube, 5.0 ml of sample urine is placed. When the volume of urine per hr is less than 50 ml, separation of organic layer after extraction is difficult, therefore, the urine should be diluted accurately to volume of 60—80 ml and 5.0 ml of this urine is taken. To the sample urine in the centrifuge tube, 5.0 ml of a buffer solution (pH 7.7) (1.0 g of NaCl dissolved in 10 ml of 0.5 M phosphate buffer, pH 7.7) and 15.0 ml of dichloroethane are added, shahen vigorously for 2 min, and centrifiged at 2000 rpm. To 13 ml of the dichloroethane layer, 1 ml of buffer solution (pH 7.7) is added, the mixture is shaken vigorously, and allowed to stand to separate the dichloroethane layer. To 12.0 ml of this dichhloroethane layer, 4.0 ml of 0.1 N sodium carbonate is added, shaken vigorously for 1 min. and the mixture is centrifuged to collect the aqueous layer. In a small glass-stoppered test tube containing 1.0 ml of a mixture of 0.5 M disodium hydrogen phosphate–0.5 M sodium dihydrogen phosphate (7; 10), 3.0 ml of the aqueous layer from the centrifugation is added, followed by 3.0 ml of dichloroethane, and the whole is shaken vigorously. After allowing the mixture to stand, the majority of the aqueous layer is removed and the residual solution is centrifuged. The dichloroethane layer is collected, taking care not to mix the water droplets, and this organic layer is used as the sample solution (T). The dichloroethane layer obtained by processing 5.0 ml of the aqueous solution of SAM (0.5 μg/ml, the standard solution) or 5.0 ml of water in the same manner as above is used for the comparative standard solution (S) or reagent solution (B).

2) Measurement of Fluorescence: Each dichloroethane solution (T, S, or B) is treated by the fluorometric determination described above, and fluorescence intensity of the solution is measured. The amount of SAM is calculated from the following equation.

$$\text{Amount of SAM in urine (ng/ml)} = \text{Amount of SAM in standard solution (ng/ml)} \times (T_F - B_F)/(S_F - B_F)$$

where B_F, S_F, and T_F are fluorescence intensity obtained by using the dichloroethane solution B, S, and T_1 respectively. B_F is measured directly after measurement of T_F and S_F.

Results and Discussion

Conditions for Fluorescence

Conditions of the solution to get a stable and strong fluorescence were as follows: A ratio of glacial AcOH to Ac_2O was 9.5: 0.5 as shown in Fig. 1, concentration of H_3BO_3 and H_2SO_4 in the measurement solution were 0.05—0.2% and 0.005—0.01 N, respectively. These conditions were the same as for salicylic acid in our preceding paper.[1]

Stability of Fluorescence——Ten mixtures of glacial AcOH–0.1% BSA solution (2: 4) containing 2×10^{-6} M SAM were prepared. The standard deviation of fluorescence intensity of the solutions was 0.4_5 and the coefficient of variation was 0.5_6%. The values obtained after heating these solutions at 40° for 1 hr. were 0.5_1, and 0.6_4, respectively.

6) T. Shibazaki, *Yakugaku Zasshi*, **90**, 413 (1970).

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Calibration Curve——Appropriate linearity was observed in the concentration range of 3×10^{-8} to 7×10^{-6} M of SAM in the solution, and quenching of fluorescence was observed clearly at 2×10^{-5} M.

Fluorescence Characteristics and Structure of SAM-Boron Complex

The maximum wavelength of the excitation spectrum of the fluorescence of the measurement solution for SAM was at 326 nm and that of the fluorescence spectrum was at 391 nm (Fig. 2). Maximum of absorption spectrum of this solution was at 326 nm, agreeing with the maximum wavelength of the excitation. This solution not containing H_3BO_3 showed maximum absorption at 305 nm and the solution showed hardly any fluorescence (Fig. 3). Bonding

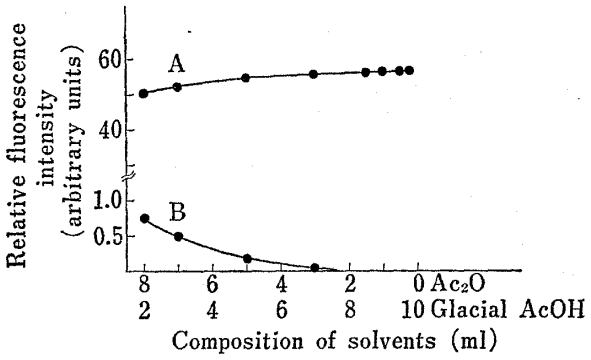


Fig. 1. Effect of Solvents on Fluorescence Intensity

A: Concentration of salicylamide, 2×10^{-6} M.
B: Concentration of salicylamide, 0.
Heating time, 30—50 min at 26°.
Concentration of H_3BO_3, 0.1%.
Concentration of H_2SO_4, 0.01 N.

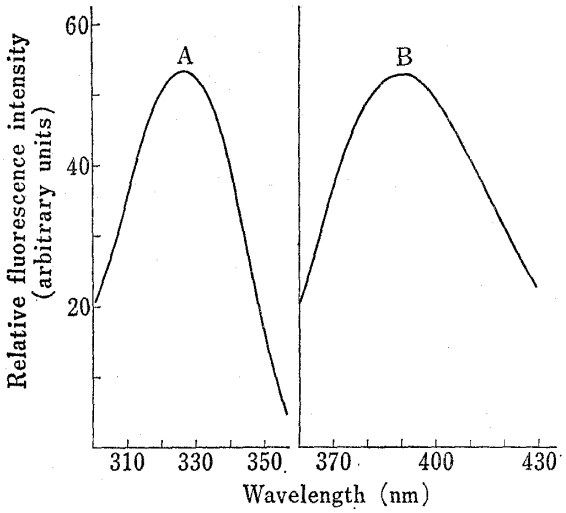


Fig. 2. Excitation and Fluorescence Spectra of Salicylamide Boron Chelate

A: excitation spectrum, emission 388 nm
B: fluorescence spectrum, excitation 328 nm
Salicylamide (5×10^{-6} M) in a mixture of 2.0 ml of glacial AcOH and 4.0 ml of BSA solution

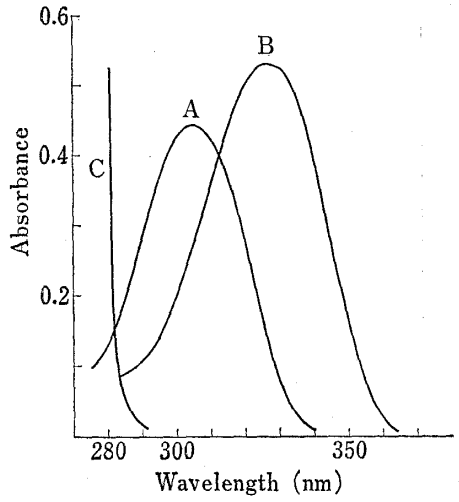
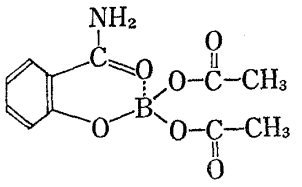


Fig. 3. Absorption Spectra

A: salicylamide (1×10^{-4} M) in glacial AcOH
reference: glacial AcOH.
B: salicylamide (1×10^{-4} M) in BSA solution
reference: BSA solution.
C: BSA solution.
reference: water.

Table I. Analysis of Salicylamide Boron Chelate



Calcd. as $C_{11}H_{12}BNO_6$ $C_7H_7O_2N$: H_3BO_3: CH_3COOH (1:1:2)[a]		Analysis (%) Found	
C	H	C	H
49.8	4.6	49.5	4.7

a) Molar ratio of salicylamide, boric acid, and acetic acid in the molecule was determined by continuous variation method, ultraviolet spectrophotometry, and titrimetry with sodium hydroxide solution.

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ratio of SAM and boron was calculated by the continuous variation method and was found to be 1:1. Elemental analysis values and measurement of the amount of SAM, H_3BO_3, and acetic acid in the crystals of the isolated reaction product revealed their composition ratio to be 1:1:2 (Table I). These results suggested that this fluorescence reaction is due to the chelation of the same type, as in the case of salicylic acid esters reported previously.[7]

Conditions for Determination of Unchanged SAM in Human Urine

Separatory Extraction of SAM——Dichloroethane, $CHCl_3$, CCl_4, and ethyl acetate were examined as the solvent for extraction of SAM. Dichloroethane was found to be the most suitable solvent for extraction from urine and the metabolites of SAM were not extracted into this solvent. Distribution of SAM in dichloroethane: water was 83:17.

Fleckenstein and others[3] used a phosphate buffer (pH 7.0) to extract unchanged SAM from human plasma with dichloroethane but, in the present experiment, optimal pH for extraction from human urine was newly examined. An equal volume of dichloroethane and aqueous solutions of SAM of various pHs was shaken, 4.0 ml of BSA solution was added to 2.0 ml of the separated dichloroethane layer, and fluorescence intensity of the reaction mixture was measured. Uniform fluorescence intensity was found in aqueous solution of pH 2—8.0, but the intensity decreased by about 6% in that of pH 8.3. On the other hand, in the urine obtained after ingestion of SAM, fluorescence intensity decreased with the rise of pH at the time of extraction, but uniform values were obtained in the pH range of 7.2—7.9. In these pH range, SAM added to the urine was extracted into dichloroethane, same as from the aqueous solution, but no salicylic acid extracted.

Urine Blank and Recovery Rate——Fluorescence intensity was measured by above described method (*cf.* experimental) using an aqueous solution containing 0.1 μg/ml of SAM, 3 kinds of urine from men not ingesting SAM (U_1, U_2, and U_3), and the same kinds of human urine added with SAM to a concentration of 0.1 μg/ml (T_1, T_2, and T_3). With the sensitivity of the recording fluorescence spectrophotometer set 10, the values were 14.5 for B_F, 15.8 for U_{1F}, 14.4 for U_{3F}, 12.1×10 for S_F, 12.0×10 for T_{1F}, 12.2×10 for T_{2F}, 11.7×10 for T_{3F},

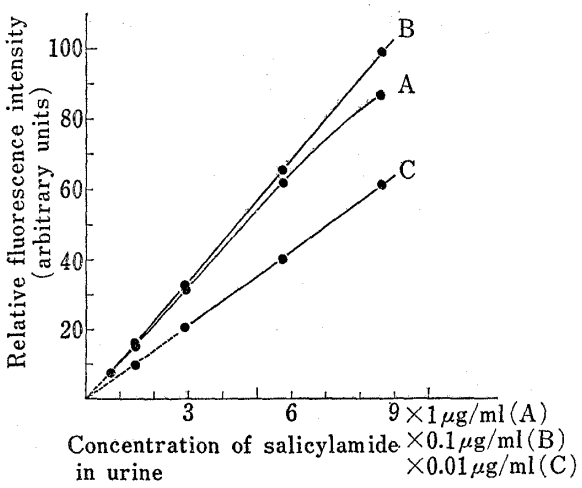

Fig. 4. Calibration Curves for Salicylamide in Urine

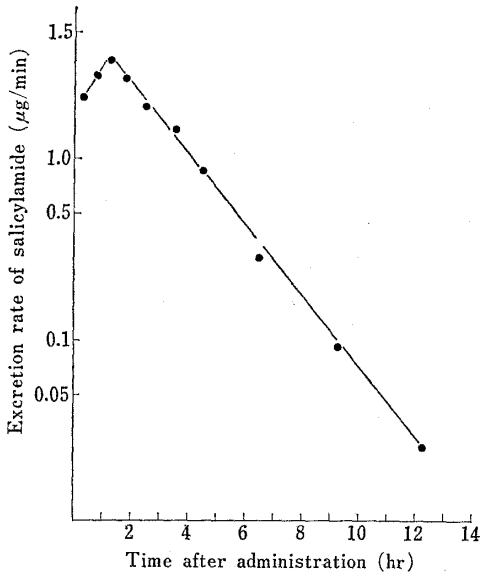

Fig. 5. Urinary Excretion Rate of Salicylamide after Oral Administration of Salicylamide (1.0 g) in Man

7) T. Shibazaki and S. Yoshioka, *Yakugaku Zasshi*, **94**, 1585 (1974).

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where B_F is the value of reagent blank obtained by using water, S_F is the value measured with aqueous solution of SAM, U_{1F}, U_{2F}, and U_{3F}, are the values with U_1, U_2, and U_3, respectively, and T_{1F}, T_{2F}, and T_{3F} are the values measured with respective urine samples added with SAM. These results indicate that the reagent blank can be used in place of urine blank for the measurement of SAM in urine.

Fluorescence intensity of the comparative standard solution obtained from aqueous solution of 0.1 μg/ml of SAM was $S_F-B_F=106.5$, and that of the urine added with the same quantity of SAM was $T_{1F}-U_{1F}=104.2$, $T_{2F}-U_{2F}=107.2$, and $T_{3F}-U_{3F}=102.6$. Recovery rate against the fluorescence intensity of the comparative standard solution was 98, 101, and 96%, respectively, and these values seemed satisfactory.

Calibration Curve and Precision——Urine samples added with 0.014—8.6 μg/ml of SAM was submitted to the determination as above (*cf.* experimental) and the calibration curve thereby obtained, as shown in Fig. 4, indicated linearity in the range of 0.03—5 μg/ml of SAM.

The coefficient of variation from 8 urine samples added with 0.55 μg/ml of SAM was 1.1.

Determination of SAM in Human Urine

One gram of finely powdered SAM is administered orally together with 250 ml of water to healthy man subject, who have fasted for at least 8 hr. At 1 hr intervals, the subject is made to drinke 50 ml of water, a light meal after 4 hr, and then allowed to spend the day normally. The urine is collected from 0.5 hr after oral administration periodically, and the amount of unchanged SAM is measured by the method described above. The result of this measurement is given in Table II, and excretion rate of SAM calculated from the data in Table II gave a first-order pattern after 1 hr shown in Fig. 5.

Table II. Urinary Excretion of Salicylamide after Oral Administration of Salicylamide (1.0 g) in Man

Time (min)	Amount of urine (ml)	Amount of unchanged salicylamide in urine (μg)
0— 30	79	64.8
60	110	85.5
90	37	105.3
125	35	96.8
180	115	107.7
243	40	90.1
300	70	49.7
480	100	51.9
630	80	14.2
840	70	5.4
		Total 671.4

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