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Fluorometric Determination of Ampicillin[1)]

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Aminobenzylpenicilloic acid, which is transformed from ampicillin readily in alkaline solution, forms an intensive and reproducible fluorescent product in neutral solution containing mercuric chloride under the condition of 40°. The fluorescent product has uncorrected excitation and emission maxima at 340 and 420 nm, respectively, and this compound is readily extracted into ethyl acetate and chloroform from acidic and neutral media and then can be back extracted into alkaline media. An assay product based on these observations permits detection of less than 0.1 μg/ml of ampicillin and/or aminobenzylpenicilloic acid. The fluorescent product is not formed from ampicillin directly, so that the direct separate measurement can be made of aminobenzylpenicilloic acid as well as unchanged ampicillin in aqueous solution, urine and blood.

Several methods for the quantitative determination of ampicillin (α-aminobenzylpenicillin, AB-PC) in aqueous solution have been described. These utilize techniques such as ultraviolet (UV) spectrophotometry,[3–5)] and hydroxamate color formation.[6)] Because of relative insensitivity of these methods, procedures based on microbiological assay[7,8)] have been used mainly for detection of AB-PC as well as many other penicillins, at the low concentrations encountered in biological fluids following therapeutic doses of the antibiotics.

Studies of the transformation product of AB-PC were reported in recent years,[9,10)] but the apparent metabolite of AB-PC in man has not been identified. And to date, in man, α-aminobenzylpenicilloic acid (AB-PA) that the β-lactam portion was cleaved, has been assumed[10)] as the major metabolite as well as other penicillin derivatives.[11)]

Recently, Jusko[10)] described a fluorometric method of total AB-PC in biological fluids. Whearas measurement can be made of ampicillin degradation product (AB-PA) as well as the unchanged antibiotic in biological fluids by combining this fluorometric and microbiological methods, the direct method of separatory determination of unchanged AB-PC and AB-PA has not been reported, to date.

An intensive and reproducible fluorescent product could be formed from AB-PA in the neutral solution containing mercuric chloride ($HgCl_2$) under the condition of 40°. In the alkaline solution, AB-PA was formed easily from AB-PC.[12)] This observation led to the development of a sensitive separatory determination for AB-PC and AB-PA in the aqueous solution, urine, and blood.

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Experimental

Materials and Reagents——Anhydrous form of AB–PC was used (potency: 998 μg/mg). AB–PC solutions were prepared freshly, befor the experiment. Pivampicillin, amoxicillin, potassium hetacillin, and potassium penicillin-G were obtained commercially. All the reagents used in the experiment were of special grade, and was freshly prepared with redistilled water.

As a standard fluorescence solution, stock solution containing 10 μg/ml of quinine sulfate was prepared with 0.1N sulfuric acid, and this solution was adequately diluted with 0.1N sulfuric acid before the experiment.

Apparatus——Fluorescence intensity was measured by a Hitachi spectrofluorometer, 203, equipped with Xenon lamp.

Method of Separatory Determination of AB-PC and AB-PA

Procedure 1——Method in Aqueous Solution: According to the procedure shown in Chart 1, a 1 ml aliquot of sample, if necessary, diluted adequately was placed in a brown test-tube. In the measurement of the total AB-PC (AB-PC+AB-PA, c_1), 0.5 ml of 1N NaOH was added to the sample solution in the brown test-tube and the mixture was allowed to stand for 5 minutes, and 0.5 ml of 1N HCl was then added. To this mixture, 1 ml of 0.04% (w/v) $HgCl_2$ solution prepared with pH 2.5 buffer solution (citric acid–HCl–NaOH) was added. After 5 minutes, 6 ml of phosphate buffer solution (pH 6.0) was added. A solution of the fluorescent product was obtained by warming this mixture for 20 minutes at 40°, and after standing for 10 minutes in room temperature fluorescence intensity of sample solution and reagent blank were measured at an excitation wavelength of 340 nm and an emission one of 420 nm, setting the intensity of fluorescence standard solution to 100 unit. In the measurement of AB-PA (c_2), 1 ml of aqueous water instead of NaOH and HCl was added to the sample solution in the another brown test-tube. And then the mixture was treated following the procedure mentioned in total AB-PC (AB-PC+AB-PA) determination. Consequently, the substraction of c_2 from c_1 gives the concentration of unchanged AB-PC.

Procedure 2——Method in Urine Sample: According to the procedure shown in Chart 1, a 1 ml aliquot of sample solution, if necessary, diluted adequately with redistilled water was placed in a brown test-tube and these sample solutions were treated following the procedure 1 to measure the total AB-PC (AB-PC+AB-PA) and AB-PA. To 9 ml of the solution after warming at 40°, 6 ml of ethyl acetate saturated with redistilled water was added, and the mixture was vigorously shaken for 2 minutes and centrifuged four milliliters of the organic layer was then added to 6 ml of 1/7M borate buffer (pH 13.0: sodium borate–NaOH).

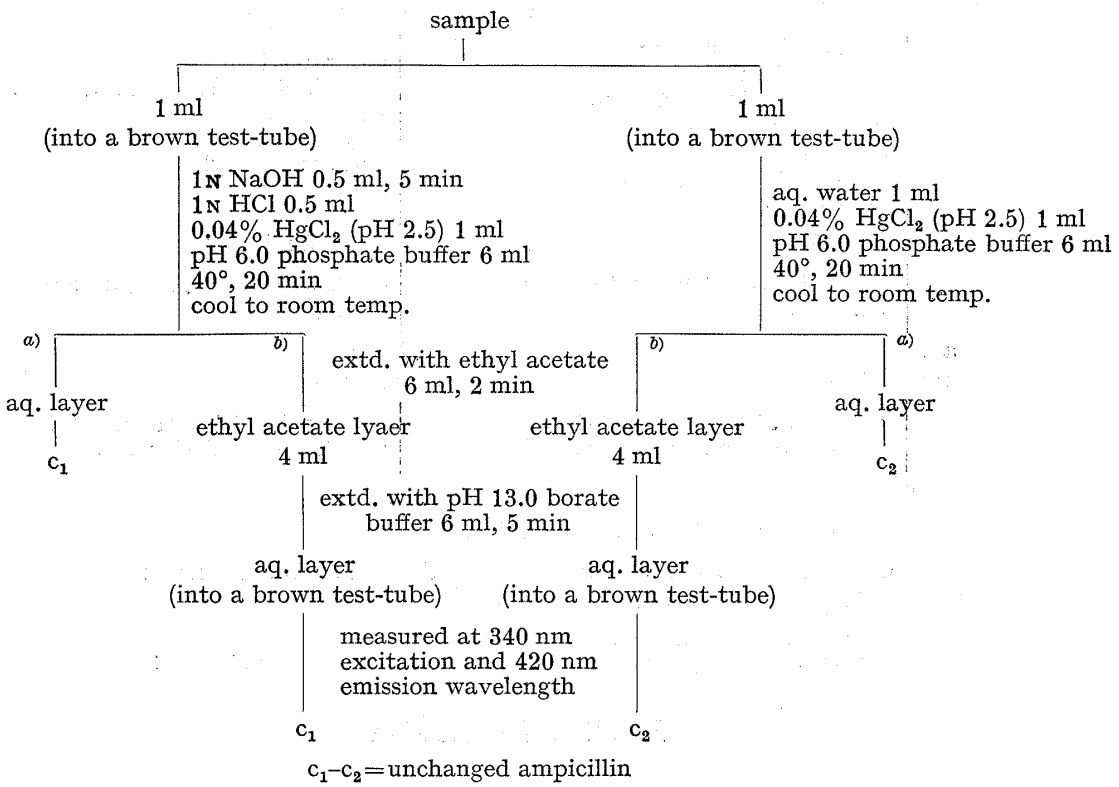

Chart 1. Method of Separatory Determination of Ampicillin and Aminobenzylpenicilloic Acid in aq. Solution and Urine

a) determination method in aq. solution, *b*) determination method in urine
c_1: amt. of total ampicillin in 1 ml of sample solution
c_2: amt. of aminobenzylpenicilloic acid equivalent to ampicillin in 1 ml of sample solution

And the mixture was shaken for 5 minutes and centrifuged. Aqueous layer of the fluorescent product was then placed into a brown test-tube. And the total AB-PC (c_1) and AB-PA (c_2) were measured respectively. Consequently, the substraction of c_2 from c_1 gives the concentration of unchanged AB-PC.

Procedure 3——Method in Whole Blood Sample: The procedure was shown in Chart 2. 0.4 ml of whole blood sample was added to 4 ml of aqueous water in a 10 ml glass-stoppered centrifuge tube. 3 ml of 10% trichloroacetic acid (TCA) was added to this hemolyzed blood sample solution and the mixture was centrifuged to obtain the clear supernatant. In the determination of total AB-PC (AB-PC+AB-PA, c_1), 3 ml aliquot of the supernatant solution, if necessary, diluted adequately with TCA solution (TCA: H_2O= 3: 4) was pipetted into a brown test-tube containing 0.5 ml of 2N NaOH and the mixture was allowed to stand for 5 minutes, and 0.5 ml of 2N HCl was then added. To this mixture, 1 ml of 0.1% (w/v) $HgCl_2$ (pH 2.5) was added. After 5 minutes, 2 ml of 2/3M Na_2HPO_4 solution was added to the mixture to adjust pH of the mixture 6.2 ± 0.1. The mixture was then warmed for 20 minutes at 40°. And then, 6 ml of ethyl acetate saturated with redistilled water was added, and the mixture was vigorously shaken for 2 minutes and centrifuged. Five milliliters of the organic layer was then added to 6 ml of 1/7M borate buffer solution (pH 13.0) and the mixture was shaken for 5 minutes and centrifuged. A solution of the fluorescent product was placed into a brown test-tube and measured at wavelengths described above.

In the procedure for the determination of AB-PA in blood sample solution (c_2), to 3 ml aliquot of sample was added 1 ml of aqueous water instead of NaOH and HCl in a brown test-tube, and 1 ml of 0.1% (w/v) $HgCl_2$ was then added. After 2 ml of 1/2M Na_2HPO_4 was added to the solution, the reaction mixture was treated following the procedure mentioned in total AB-PC determination. Consequently, the substraction of c_2 from c_1 gives the concentration of unchanged AB-PC.

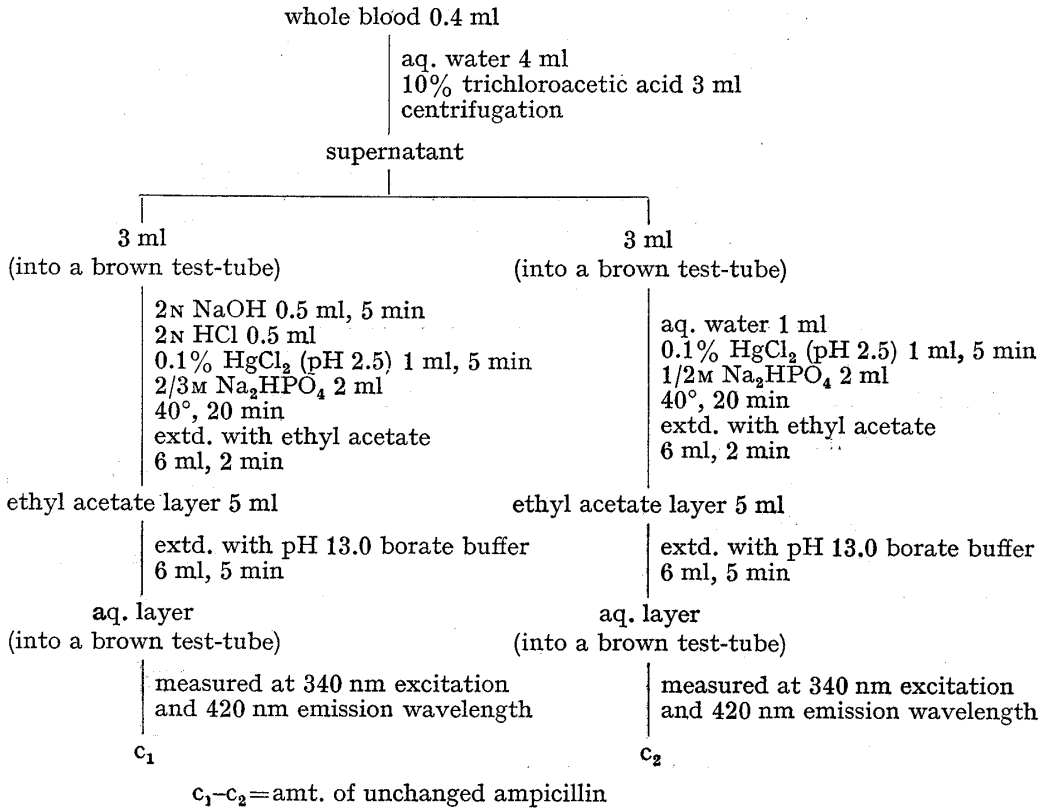

Chart 2. Method of Separatory Determination of Ampicillin and Aminobenzylpenicilloic Acid in Blood

c_1: amt. of total ampicillin in 0.4 ml of blood
c_2: amt. of aminobenzylpenicilloic acid equivalent to ampicillin in 0.4 ml of blood

Result and Discussion

Properties of the Fluorescent Product from AB-PC

A intensive and reproducible fluorescent product was obtained from AB-PA, which is formed from AB-PC in alkaline solution easily, in the neutral solution containing $HgCl_2$ solution under the condition of 40°. The excitation and emission spectra of this product are shown

in Fig. 1. Consequently, all fluorescence measurement in this study were carried out at the excitation maximum at 340 nm and the emission maximum at 420 nm.

Effect of the Temperature on the Fluorescence Intensity——The rate of formation of the fluorescent product from AB-PC or AB-PA at 40° and room temperature in pH 6.0 buffer solution was studied according to the procedure 1 (Fig. 2). In the experiment that AB-PA was warmed under the condition of 40°, a constant value was obtained between 20 and 60 minutes. On the other hand, the fluorescent product was not formed from AB-PC in this condition. Thus it was shown that the fluorescent product was obtained by warming the AB-PA solution for 20 minutes at 40° after transformation of AB-PC to AB-PA in alkaline solution.

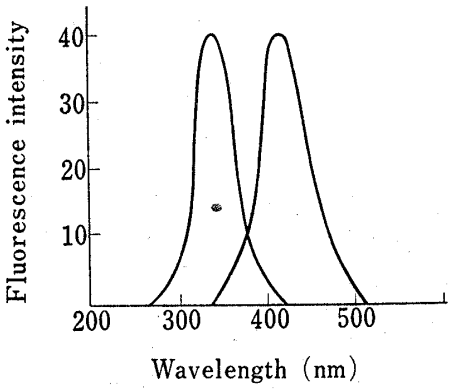

Fig. 1. Spectrophotofluorometric Excitation (340 nm) and Emission (420 nm) Spectra for the Fluorescent Product of Ampicillin

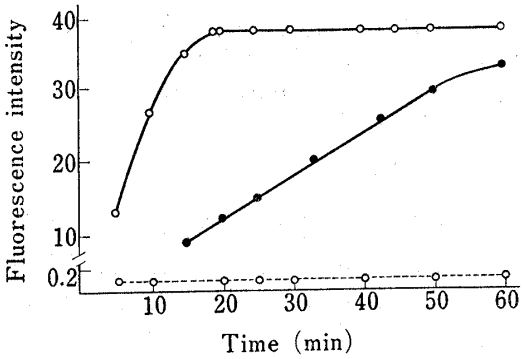

Fig. 2. Relationship between Fluorescence Intensity and Standing Time at 40° and Room Temperature

—○—: aminobenzylpenicilloic acid (40°)
—●—: aminobenzylpenicilloic acid (room temp.)
---○---: ampicillin (40°)

Effects of the Concentration and pH of $HgCl_2$ Reagent on the Fluorescence Intensity——The effects of concentrations of $HgCl_2$ reagent on the maximum intensity of fluorescence from AB-PC in aqueous solution and urine sample were investigated according to the procedure 1 and 2. As the result shown in Fig. 3, the maximum and the constant fluorescence intensity was obtained at the concentration range of 0.03—0.06% (w/v) of $HgCl_2$ reagent.

And in the experiment in blood sample solution (procedure 3), the maximum fluorescence intensity was obtained at the concentration range of 0.10—0.15% (w/v) of $HgCl_2$ reagent.

Furthermore, the effects of pH of the $HgCl_2$ solution on the fluorescence intensity were investigated. It was found that the fluorescence intensity is greatest and constant at acidic condition (pH 1—3.5). Consequently, the $HgCl_2$ reagent was prepared with pH 2.5 buffer solution (citric acid–HCl–NaOH).

Effects of pH on the Formation of the Fluorescent Product and the Fluorescence Intensity——To investigate the effect of pH on the formation of the fluorescent product, various pH sample solutions (pH 2—10) were warmed for 20 minutes at 40°. As the result shown in Fig. 4, the maximum fluorescence intensity was obtained over the range of 5.9—6.3.

And to increase the sensitivity of the fluorescence intensity, the pH-fluorescence profile was obtained in various pH sample solutions (Fig. 5). According to this results, all measurements of the fluorescence intensity in urine and blood sample solutions were carried out in alkaline solution to increase the sensitivity after warming the reaction mixtures in neutral media at 40°.

Furthermore, the fluorescence intensity was very stable up to 30 minutes.

Solvent Extraction——In order to separate the fluorescent product from the interfered materials in the body fluids, the extractibility of the fluorescent product in various pH media with several organic solvents were studied. The compound is readily extracted into ethyl

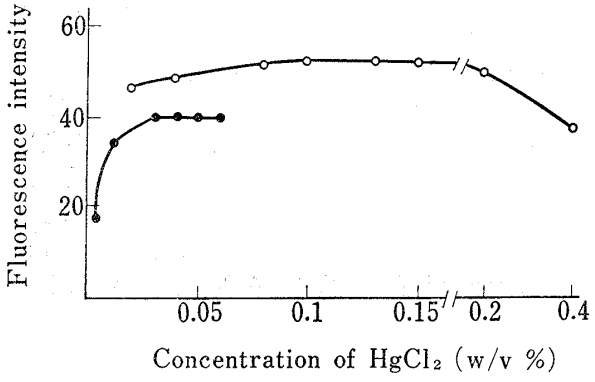

Fig. 3. Effects of Concentration of $HgCl_2$ Reagent on the Fluorescence Intensity

—●—: in aqueous solution and urine sample (procedure 1 and 2)
—○—: in blood sample (procedure 3)

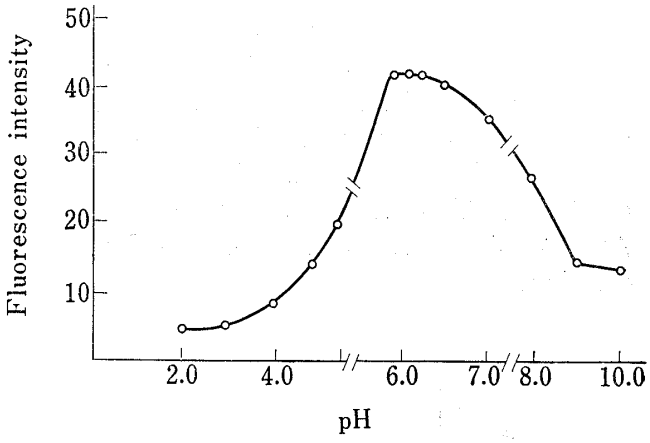

Fig. 4. Relationship between Fluorescence Intensity and Various pH Reaction Mixtures at 40°

acetate, chloroform and isoamyl alcohol from acidic and neutral solution. And then the product can be subsequently reextracted into alkaline solution from organic solvents. On the other hand, AB-PC itself could not be extracted from aqueous solution into any of the solvent tested, especially into ethyl acetate. Consequently, ethyl acetate was suitable for the extraction of the fluorescent product from the body fluids. Furthermore, according to the results shown in Fig. 5 and the reextractibility of the fluorescent product, pH 13.0 buffer solution (1/7M sodium borate–NaOH) was used as the reextracting medium. By means of this method, the blank estimation in the fivefold diluted urine sample and blood sample was not interfered.

There was a linear relationship between AB-PC concentration and fluorometric response in the range of 0.04—0.4 μg/0.4 ml whole blood.

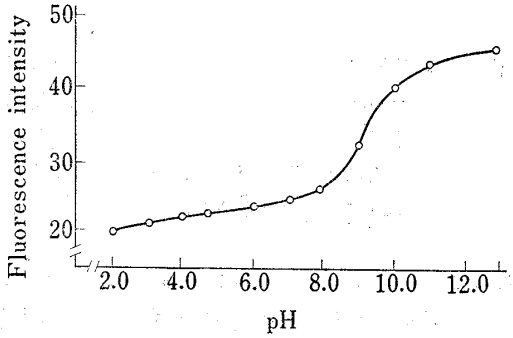

Fig. 5. Relationship between Fluorescence Intensity and Various Final pH Sample Solutions Measured

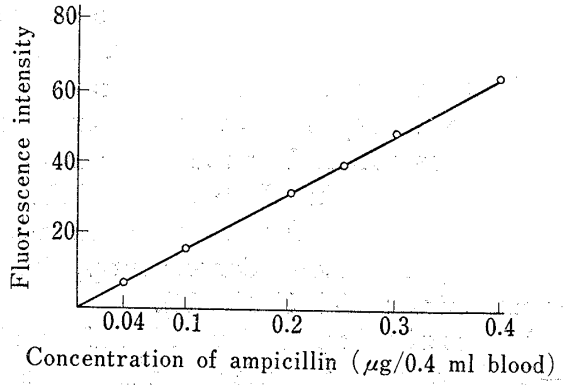

Fig. 6. Fluorometric Standard Curve Obtained for Ampicillin Added to Pooled Human Whole Blood

Recovery Test of AB-PC and AB-PA

A mixture containing a known amount of every standard substance in aqueous solution, human urine and human blood was separately determined following the procedure in Chart 1 and Chart 2, respectively. The results are given in Table I, which agreement between added amount and found value is reasonable. Thus this separatory determination method was found to be applicable for the aqueous, the urine and the blood samples containing AB-PC and AB-PA.

Table I. Recovery Test of Ampicillin and Aminobenzylpenicilloic Acid

Sample	Added (μg) AB-PC			Added (μg) AB-PA[a]	Recovery (%)[b] AB-PC	Recovery (%)[b] AB-PA
Aq. soln.	2.0	5.0	10.0	2.0	99.0—101.8 (3)	98.8—102.9 (3)
	2.0	5.0	10.0	5.0	99.5—102.2 (3)	98.8—100.8 (3)
	2.0	5.0	10.0	10.0	98.8—101.0 (3)	98.3—100.7 (3)
Urine		20.0		2.0	99.3— 99.7 (4)	99.3—101.3 (4)
		40.0		2.0	97.9— 98.3 (4)	100.7—101.3 (4)
Blood[c]		2.0		0.2	96.8—100.8 (4)	103.0—104.8 (4)
		2.0		1.0	98.5— 99.0 (2)	99.7—100.6 (2)
		2.0		5.0	100.4—101.2 (2)	99.7—100.0 (2)

a) shown as ampicillin equivalent
b) Range of recovery (%) is shown.
c) Sample was added to 0.4 ml human whole blood.
Experiment no. is given in parentheses

Application of This Method for Other AB-PC Derivatives

The assay procedure was studied, using several penicillin derivatives as shown in Table II. As expected, pivampicillin which is equivalent of AB-PC concentration gives the same degree of fluorescence intensity. Amoxicillin and hetacillin formed a sufficient amount of fluorescent product. On the other hand, benzylpenicillin, which lacks the α-amino group of AB-PC, forms no fluorescent product. These data show that the aminobenzyl group is necessary for the formation of the fluorescent product.

Table II. Relative Amount of Fluorescent Material formed from Various Penicillin Derivatives

Penicillin derivatives (5 μg/ml)	Relative fluorescence intensity
Ampicillin	100
Pivampicillin	100
Amoxicillin	50
Hetacillin	35
Penicillin-G	0

Table III. Urinary Excretion of Ampicillin and Aminobenzylpenicilloic Acid after administered orally 250 mg of Ampicillin to Male Subject

Subject No.	Substance recovered	Amount excreted in urine (mg)[a] hr[b] 0—1	1—2	2—3	3—4	4—5	5—6	Total by 6 hr	% of dose 0—6 hr
1	ampicillin	5.84	26.38	25.38	17.72	9.55	3.60	88.47	35.39
	AB-PA	0.23	1.87	2.81	2.45	1.88	1.09	10.33	4.13
2	ampicillin	3.26	11.31	15.04	7.89	7.95	4.32	49.77	19.91
	AB-PA	0.20	1.06	1.99	1.50	1.64	1.13	7.52	3.01
3	ampicillin	0.44	30.62	41.42	22.65	8.99	5.09	109.22	43.69
	AB-PA	0.00	1.46	2.63	2.09	1.26	0.80	8.24	3.30
4	ampicillin	0.89	17.09	24.23	13.20	9.08	4.79	69.27	27.71
	AB-PA	0.05	1.03	2.10	1.51	1.36	0.88	6.94	2.77

These values were obtained following this new determination method for ampicillin.
a) shown as ampicillin equiv.
b) time intervals of sample collection after administration

Quantitative Study on AB-PC and AB-PA in Human Urine

As shown in Table III, AB-PC and AB-PA in urine samples were separately determined after dosing 250 mg of AB-PC to human subjects. The results are shown in Table III. In the urine during 6 hours after doses of the drug, the amount of unchanged AB-PC recovered was 20—45% of the dose. The amount of AB-PA recovered, on the other hand, was only 3—4% of the dose. Thus it was found that the small amount of AB-PA in the presence of the large amount of AB-PC could be measured directly and rapidly by this fluorometric method.