Determination of β-Lactam Antibiotics in Water by Fluorescence Quenching of Mercurochrome,[1] and Application for Simple Investigation of Potency

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The fluorescence quenching reaction between fluorescein mercury or halogeno-fluorescein mercury compounds (fl. Hg, 2,7- or 2,4-dichloro-fl.Hg, 3′,4′,5′,6′-tetrachloro-fl.Hg, mercurochrome) and β-lactam antibiotics (ampicillin (AB-PC) and cephalexin (CEX)) was investigated, and mercurochrome was selected for the detection of β-lactam antibiotics; the detection limit was about 0.8 μg/ml.

A fluorimetric assay of β-lactam antibiotics was established by measuring the fluorescence of mercurochrome and mercurochrome–β-lactam antibiotics solutions in weakly basic media to determine the degree of fluorescence quenching. The maximum emission wavelength of mercurochrome solution was at 544 nm with excitation at 470 nm. The calibration graphs were linear over the ranges of about 0—6 μg/ml β-lactam antibiotics penicillins (AB-PC, penicillin G, sulbenicillin, amoxicillin, cyclacillin, oxacillin, hetacillin and piperacillin) and cepham antibiotics (CEX, cefazolin, cephaloglycin, cephaloridine and cefpyramide), and the relative standard deviation was 2.7% for 1.4 μg/ml of AB-PC ($n=5$).

This fluorescence quenching reaction between mercurochrome and β-lactam antibiotics was applied in a survey of decomposition and remaining potency of β-lactam antibiotics.

Keywords fluorimetry; mercurochrome; fluorescence quenching; β-lactam antibiotic; fluorescein derivative; mercury compound; potency

Penicillins, a family of β-lactam antibiotics containing a sulfur atom, are long-established and effective antibiotics. For the spectrophotometric determinations of β-lactam antibiotics, numerous direct complex-forming reaction systems of β-lactam antibiotics with metal ions such as iron (III), mercury (II), copper (II), vanadium (V) and/or various organic reagents have been studied[2a–e]; for example, Haginaka *et al.*[2d,e] reported the complex-forming reaction among mercuric chloride, 1,2,4-triazole or imidazole and penicillin. However, the use of organic metal compounds such as organo mercury and organotin compounds has not been fully investigated.

Fluorescein mercury (fl.Hg) or halogeno-fluorescein mercury (X-fl.Hg) compounds have already been used[3a–i] for the fluorimetric determination of sulfur compounds such as thiourea derivatives or long-chain quaternary alkylamine compounds such as *N*-hexadecylpyridinium chloride (HPC). Previously, we have reported[4] that the color or fluorescence quenching reaction between β-lactam antibiotics such as penicillin and X-fl.Hg compounds can be applied to the assay of β-lactam antibiotics, analogously to the complex-forming reaction using mercuric chloride and an organic reagent such as imidazole.

In this paper, the fluorescence properties of various X-fl.Hg compounds were examined, and a simple analytical method for β-lactam antibiotics was developed by measuring the difference of fluorescence intensities between mercurochrome and mercurochrome–β-lactam antibiotic solutions. This method was applied to examine the decomposition and remaining potency of β-lactam antibiotics.

Experimental

Apparatus and Reagents The fluorimetric measurements were carried out on Hitachi model 3000 and Shimadzu model RF-500 fluorometers, using matched 10-mm silica cells. A Hitachi-Horiba model F-7AD pH meter, equipped with a glass and colomel combined electrode was used for all pH measurements.

A 0.1% mercurochrome solution was prepared by accurate dilution of a 0.5% stocked mercurochrome solution (Yamayoshi Chemical Co., Ltd.). Also, 0.1% fl.Hg and X-fl.Hg solutions, *i.e.*, fl.tetrahydroxymercury compound, 2,7-di- or 2,4-dichloro-fl.dihydroxymercury compound (2,7-Cl.fl.Hg, 2,4-Cl.fl.Hg) and 3′,4′,5′,6′-tetrachloro-fl.tetrahydroxymercury compound (3′,4′,5′,6′-Cl.fl.Hg), were prepared by dissolving these Hg compounds in 1 N sodium hydroxide solution according to previous reports.[3h,i,4] Aqueous solutions of ampicillin (AB-PC), amoxicillin (AM-PC), sulbenicillin (SB-PC), cyclacillin (CC), hetacillin (HC), oxacillin (OC) and piperacillin (PC) as penicillins, and cephalexine (CEX), cephaloridine (CED), cephaloglycin (CEG) and cephpramide (CEP) as cepham antibiotics, were prepared by dissolving AB-PC, AM-PC, SB-PC and OC, HC, CC, PC CEX, CED, CEP sodium or potassium salt in water, respectively. These solutions were prepared before just use, and were stored in a dark, cold place. A 1.0% aqueous solution of polyvinyl alcohol (PVA) was prepared by dissolving PVA ($n=500$, Kishida Chemical Co., Ltd.) in hot water without further purification. Sörensen buffer solution (5.0×10^{-2} M borax and 1.0×10^{-1} M sodium hydroxide, pH 10) was used for pH adjustments. The surveys of potency for β-lactam antibiotics were carried by the standard method (minimum requirements of antibiotics products in Japan) by using the paper disk method, with *Bacillus subtilis* PCI-219 for AB-PC and *Bacillus subtilis* ATCC 6633 for CEX. All other reagents and materials were of analytical reagent grade, and deionized water was used.

Detection Test In the standard procedure, 0.1 ml of a 0.1% mercurochrome solution, 0.5 ml of a 1.0% PVA solution and 1.0 ml of Sörensen buffer solution are placed in a test tube. To this solution is added a drop of β-lactam antibiotic solution containing more than about 4.0 μg, and the mixture is diluted to 5.0 ml with water. The reaction mixture is kept at 60 °C for 45 min, and its color (increasing red color) or fluorescence intensity is compared with that of a blank solution.

Fluoriphotometry In the standard procedure, a sample solution containing up to about 50—60 μg of β-lactam antibiotic (PC-G, AB-PC, AM-PC, SB-PC, CEX, CED, CEG, CEP, *etc.*) is placed in a 10 ml volumetric flask. Then 2.0 ml of a 1.0% PVA solution, 2.0 ml of Sörensen buffer solution (pH 10) and 0.25 ml of a 0.1% mercurochrome solution are added, and the mixture is diluted to 10.0 ml with water (solution A). This sample solution is kept at 60 °C for 45 min together with a blank solution (solution B), after standing at room temperature (10—25 °C) for 5 min. The difference of relative fluorescence intensities between solutions A and B (quenching, ΔF) is measured at an emission wavelength of 544 nm with excitation at 470 nm.

Relationship between Fluorescence Quenching and Potency The potency of AB-PC of CEX was investigated by the standard paper disk method (minimum requirements of antibiotic products in Japan) with

heart infusion agar medium (Eiken Chemical Co., Ltd.). The quenching of mercurochrome fluorescence by AB-PC or CEX was measured as described above.

Results and Discussion

Fluorescence Quenching Reaction and Detection Test First the fluorescence properties of X-fl. or X-fl.Hg compounds were investigated. X-fl.Hg compounds in which the 4 or 5 position of 6-hydroxy-3*H*-xanthen-3-one is occupied by an acetoxymercury or hydroxymercury moiety, still showed fluorescence, since these X-fl.Hg compounds had no inhibition of resonance, and the relative fluorescence intensity of X-fl.Hg compounds in solution was smaller than that of X-fl. in solution owing to the effect of the two heavy atoms.

Next, the color or fluorescence quenching reactions between X-fl. or X-fl.Hg compounds and β-lactam antibiotics, such as PC-G, AB-PC, SB-PC, AM-PC and CEX, were systematically investigated. No effect was observed in the case of X-fl. However, fluorescence quenching phenomena were clearly elicited between X-fl.Hg and freshly prepared β-lactam antibiotics after heating at 50—60 °C and with decomposition products of β-lactam antibiotics such as penicilloic acid[5] at room temperature. Fluorescence quenching between mercurochrome and freshly prepared β-lactam antibiotics solutions was scarcely recognized at room temperature; β-lactam antibiotics aqueous solutions were relatively stable for about a month in a dark and cold place. From the fluorescence quenching between various X-fl.Hg compounds and AB-PC, mercurochrome solution was concluded to be superior to the other X-fl.Hg compounds in terms of sensitivity, reproducibility and stability (color and fluorescence quenching reaction), as shown in Table I. Accordingly, mercurochrome (4,5-dibromo fl. monohydroxymercury, merbromine, used as a general disinfectant and fungicide) solution was chosen as an analytical agent, and the detection test for β-lactam antibiotics was investigated in a test tube. As shown in Table II, the detection test was very sensitive and simple in comparison with other methods.[2,6]

Fluorescence Spectra Figure 1 shows the excitation and emission spectra of mercurochrome solution in basic medium. The maximum excitation and emission wavelengths of mercurochrome solution were about 470 and 544 nm, respectively. Figure 2 shows the emission spectra of the mercurochrome solution (solution B) and mercurochrome–AB-PC mixed solution (solution A) in basic media. The relative fluorescence intensity of mercurochrome solution was decreased by AB-PC and the difference (ΔF) at 544 nm between solutions A and B in the standard procedure was proportional to the concentration of AB-PC. Accordingly, further investigation of the fluorimetric determination of β-lactam antibiotics was carried out at 544 nm (λ em) with 470 nm (λ ex) in basic media.

Table I. Fluorescence Quenching of Fluorescein- or Halogeno-fluorescein Mercury Compounds by AB-PC in the Standard Procedure

fl.Hg, X-fl.Hg compounds	Fluorescence[a] %	Fluorescence[a] $\lambda_{max.em}$ (nm)	Quenching[b] %	Quenching[b] $\lambda_{max.em}$ (nm)
fl.Hg	93.0	540	30.4	540
2,7-Cl.fl.Hg	100.0	540	18.5	540
2,4-Cl.fl.Hg	100.0	540	24.7	540
3′,4′,5′,6′-Cl.fl.Hg	93.5	555	30.4	550
Mercurochrome (4,5-Br.fl.Hg)	85.5	540	37.7	544

AB-PC, 35 μg/10 ml; fl.deriv.Hg, 0.25 ml of 0.1% solution; PVA, 0.1%; excitation wavelength, 470 nm; pH, 10.0. *a*) Fluorescence intensity of fl.deriv.Hg solution at maximum emission wavelength. *b*) Quenching of fluorescence of fl.deriv.Hg by AB-PC at maximum emission wavelength (ΔF).

Table II. Detection Tests of Penicillins and Cepham Antibiotics with Mercurochrome

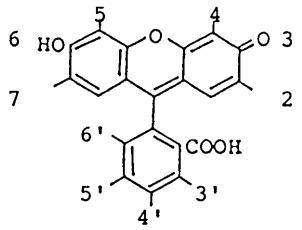
β-Antibiotic	Color: Sample	Color: Blank	Detection limit μg/ml
AB-PC	or Rd	Or	0.8
PC-G	or Rd	Or	1.0
AM-PC	or Rd	Or	1.0
SB-PC	or Rd	Or	1.5
CEX	or Rd	Or	3.5

Mercurochrome, 0.1% solution; pH 10.0; or, orangish; Rd, red; Or, orange.

Effects of pH and Surfactants Maximum and almost constant ΔF was obtained over the pH range of 9.6—10.5 with 1.5—4.0 ml of Sörensen buffer solution in the fluorescence quenching reaction.

The effect of coexisting surfactant was systematically investigated.[7] The results are shown in Table III. Among various non-ionic surfactants tested, PVA ($n=500$, 2000), gum arabic, poly-(*N*-vinylpyrrolidone) (PVP, K-15, K-30), Brij 35, Brij 58, Tween 20 (poly(oxyethylene)dodecylether), *etc.*, PVA ($n=500$) was found to give the best dispersion and stability, and maximum and almost constant ΔF was obtained by addition of 0.1% final concentration.

Effect of Mercurochrome Concentration and Stability Maximum, constant ΔF was obtained by addition of 0.1—0.4 ml of a 0.1% mercurochrome solution in a final volume of 10 ml. The fluorescence quenching reaction between mercurochrome and β-lactam antibiotics was slow at room temperature, and it was necessary to allow the mercurochrome–AB-PC solution to stand for periods up to 10 h. However, heating of solutions A and B at 60 °C for 45 min gave constant and stable ΔF (stable for at least



fl.deriv.Hg	2	4	5	7	3′	4′	5′	6′
fl.Hg_4	HgOH	HgOH	HgOH	HgOH	H	H	H	H
2,7-Cl.fl.Hg_2	Cl	HgOH	HgOH	Cl	H	H	H	H
2,4-Cl.fl.Hg_2	Cl	Cl	HgOH	HgOH	H	H	H	H
3′,4′,5′,6′-Cl.fl.Hg_4	HgOH	HgOH	HgOH	HgOH	Cl	Cl	Cl	Cl
mercurochrome	H	Br	Br	HgOH	H	H	H	H

Chart 1

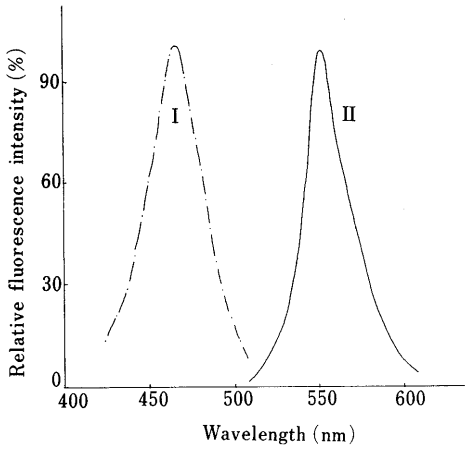


Fig. 1. Emission and Excitation Spectra of Mercurochrome Solution at pH 10

Mercurochrome, 0.15 ml of 0.1% mercurochrome solution/10 ml; PVA, 0.1%; curve I, excitation spectra with emission at 544 nm; curve II, emission spectra with excitation at 470 nm.

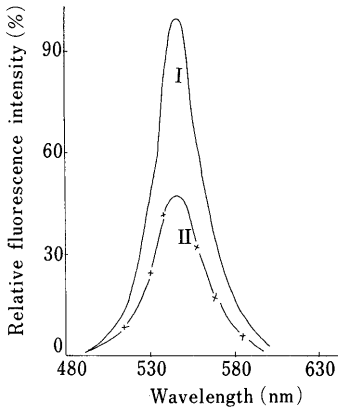


Fig. 2. Fluorescence Spectra of Mercurochrome Solution (Solution B) and Mercurochrome–AB-PC Mixed Solution (Solution A) at pH 10

Mercurochrome, 0.15 ml of 0.1% mercurochrome solution/10 ml; AB-PC, 1.4×10^{-5} M; PVA, 0.1%; curve I, solution B; curve II, solution A; excitation wavelength, 470 nm.

3 h). On the other hand, color or fluorescence quenching development was scarcely recognized in the reaction using Br-fl. and mercuric chloride, instead of mercurochrome.

Calibration Curve Calibration curves obtained by the standard procedure were rectilinear up to about 40—50 μg/10 ml (about 1.5×10^{-5} M) of PC-G, AB-PC, SB-PC, AM-PC, CC, OC, PC, and about 50—60 μg/10 ml of CEX, CEG, CEP. The sensitivity for cepham antibiotics was 1/2—1/3 of that for penicillins. Thus, the proposed method is highly sensitive (about 6 times more sensitive than the imidazole-mercury chloride method[2e]), and the relative standard deviation (reproducibility) for 14 μg of AB-PC in a final volume of 10 ml was found to be 2.7% (5 experiments).

Effect of Various Substances The effects of various ions or substances on the assay of AB-PC were investigated. Table IV shows the limiting values of foreign ions or substances which did not affect the assay (less than $\pm 3\%$ ΔF value). Caffeine or sulfisomidine did not interfere in equimolar amounts with respect to AB-PC, but organic compounds containing nitrogen, such as riboflavine and pyridoxine, gave positive errors even in small amounts. The presence of

Table III. Effect of Surfactants

Surfactants			ΔF at 544 nm
Cationic	Anionic	Nonionic	%
—	—	—	34.6
—	—	PVA (n= 500)	37.7
		(n=2000)	37.5
—	—	Tween 20	32.8
—	—	Brij 35	30.2
—	—	Brij 58	31.0
—	—	PVP (K-15)	34.4
—	—	PVP (K-30)	35.0
—	—	Gum arabic	30.4
—	—	Gelatin	3.4
—	—	Methylcellulose	36.8
—	SLS	—	34.1
HPC	—	—	36.0

AB-PC taken, 1.0×10^{-5} M; mercurochrome, 0.015%; surfactant, 0.1%; pH 10.0.

Table IV. Effect of Foreign Substances

Substance	Added as	Mol ratio Substance /AB-PC	ΔF at 544 nm %
—	—	—	41.5
NH_4(I)	Chloride	150	44.3
Fe(III)	Sulfate	5	45.6
Cu(II)	Nitrate	1	33.7
SCN^-	Potassium	2000	40.5
S^{2-}	Sodium	0.3	53.0
$S_2O_3^{2-}$	Sodium	25	20.6
Riboflavin		0.3	52.3
Pyridoxine·HCl		0.3	30.3
Chlorpromazine	HCl	2	37.4
Thiamine	HCl	5	58.6
Sulfisomizine		10	48.3
Caffeine		25	48.6

AB-PC taken, 40.5 μg/10 ml; PVA, 0.1%; pH, 10.0; mercurochrome, 0.015%.

thiocyanate, thiosulfate and iodide ions in relatively large amounts seriously interfered with the assay. Though zinc (II), cobalt (II), copper (II), iron (III) interfered in 5- to 100-fold excess over AB-PC, the interference of these metal ions could be masked by addition of nitrilotriacetic acid (NTA).

Application to Pharmaceuticals and Biological Samples The proposed method was applied to the assay of AB-PC in 1.0% AB-PC ointment. The results were as expected, and recovery was satisfactory, about 98.0—105.3%. The recoveries of AB-PC added to calf serum (Nakarai Chemical Co., Ltd.) and human urine were also examined according to the previously reported precedure,[8] and good results were obtained (recovery = 99.5%, CV = 0.8%).

Relationship between Fluorescence Quenching Development and Potency of β-Lactam Antibiotics The magnitude of fluorescence quenching between mercurochrome and β-lactam antibiotics was proportional to the concentration of β-lactam antibiotics. The relative fluorescence intensity values of mercurochrome–β-lactam antibiotics solutions varied amongst freshly prepared β-lactam antibiotics and long-stored (partially decomposed[9]) or predecomposed β-lactam antibiotics. It could be presumed that the fluorescence quenching reflected the remaining potency of β-lac-

TABLE V. Relationship between Fluorescence Intensity and Potency of β-Lactam Antibiotics

Sample	Conditions			ΔF at 544 nm %	Potency[a] (Inhibition zone) mm
	pH	Temp. (°C)	Time (min)		
AB-PC	10	60	45	0	33.0
	10	70	7	4.2	21.0
	10	70	30	14.8	11.0
	10	70	50	30.7	3.0
	10	70	60	47.2	0
CEX	10	60	45	0	39.0
	8	70	30	2.1	24.0
	10	70	30	15.5	18.0
	13	70	30	25.6	1.1
	10	70	45	40.8	0

a) Paper disk method.

tam antibiotics. Accordingly, the relation between ΔF and potency of AB-PC and CEX was studied under various conditions of pH (8, 10, 13), temperature (60 °C, 70 °C) and time (7, 30, 45, 50, 60 min). As shown in Table V, the ΔF values showed a good inverse correlation with the diameter of the inhibitory zone. Thus, the proposed method using mercurochrome is available as a simple, rapid, selective and sensitive detection test or method for assay of β-lactam antibiotics, and for confirming the potency of β-lactam antibiotics.

Conclusion

The fluorescence quenching between various X-fl.Hg compounds and β-lactam antibiotics was systematically investigated in the presence of various surfactants in basic media. The use of mercurochrome in the presence of PVA as a non-ionic surfactant was most appropriate in terms of sensitivity, reproducibility and universality. The relative fluorescence intensity difference (ΔF value) between mercurochrome solution and its solution containing β-lactam antibiotics (solutions A and B) was used for the detection test or fluoriphotometric assay of potency. The detection limit of AB-PC was 0.8 μg/ml in a test tube, and the assay range was up to 50 μg/10 ml penicillins and up to 60 μg/10 ml cepham antibiotics. The proposed fluoriphotometric method is about 6 times more sensitive than the imidazole–mercury (II) method,[2e] and is simple, rapid and selective.

Since of the magnitude of color development or fluorescence quenching between mercurochrome and β-lactam antibiotics reflected the potencies of the β-lactam antibiotics, the proposed method should be useful for the separative assay of total and decomposed β-lactam antibiotics; *i.e.*, total β-lactam antibiotics can be determined after heating at 60 °C for 45 min, and decomposed β-lactam antibiotics can be assayed at room temperature. Thus, the proposed method may be applied to a simple and rapid examination of the remaining potency of β-lactam antibiotics.

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