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Fluorometric Determination of Cephalexin in Urine[1]

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An intensively fluorescent product was formed in alkaline solution by hydrolysis of cephalexin at elevated temperature. The fluorescent product gave excitation and emission maxima at 355 and 435 nm, respectively. It was readily extracted into ethyl acetate and a acetone–chloroform mixture from an acidic solution and then extracted back into an alkaline solution. Using this new method, urinary excretion behaviors in human volunteers after oral administration of cephalexin capsule were investigated.

Several methods for the quantitative determination of cephalexin in aqueous solutions have been described.[3-6] These utilize techniques such as ultraviolet spectrophotometry,[3] polarography,[4] and infrared spectrophotometry.[5] Because of relatively poor sensitivity of these methods, procedures based on microbiological assay[6] have been used mainly for determination of cephalexin as well as many other cephalosporins at the low concentrations encountered in biological fluids following therapeutic doses of the antibiotics.

Recently it was found that a strongly fluorescent product was formed from ampicillin[7] by acid hydrolysis at elevated temperatures. Cephalexin, however, yielded a small amount of the fluorescent material because β-lactam ring in cephalexin molecule is more resistant to acid hydrolysis.[8] The authors observed that intensively fluorescent product was formed from cephalexin in alkaline solution at elevated temperatures. This observation led to the development of a sensitive assay method for cephalexin in aqueous solutions and also in urine samples. Using this new assay method, urinary excretion behaviors of cephalexin following oral administration of the antibiotic were also investigated.

This study has been undertaken as a part of our investigation on β-lactam antibiotics. Fluorometric determination of ampicillin and aminobenzylpenicilloic acid has been reported.[9]

Experimental

Materials and Reagents——Monohydrate form of cephalexin (potency: 959 mcg/mg), cephaloglycin dihydrate, cephaloridine, and sodium cephalothin were supplied by Shionogi Seiyaku Co. Ampicillin (anhydrous) was supplied by Takeda Chemical Industries. As the standard fluorescence solution, a stock solution containing 10 mcg/ml of quinine sulfate was prepared in 0.1 N sulfuric acid and this solution was adequately diluted with 0.1 N sulfuric acid before the experiment. Buffering reagents and solvents were of reagent grade.

Apparatus——Fluorescence intensity was measured by a Hitachi Spectrofluorometer Model 203 equipped with a Xenon lamp.

1) This work was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
2) Location: *Kita-12, Nishi-6, Kita-ku, Sapporo, 060, Japan.*
3) L.P. Marrelli in "Cephalosporins and Penicillins," E.H. Flynn, Ed., Academic Press, New York, 1972, pp. 630—632.
4) E.J. Benner, *Antimicrob. Ag. Chemother.*, **1971**, 201.
5) B. Casu and P. Ventura, *J. Pharm. Sci.*, **63**, 211 (1974).
6) P.W. Muggleton, C.H. O'Callaghan, R.D. Foods, S.M. Kirby, and D.M. Ryan, *Antimicrob. Ag. Chemother.*, **1968**, 353.
7) W.J. Jusko, *J. Pharm. Sci.*, **60**, 728 (1971).
8) T. Yamana, A. Tsuji, K. Kanayama, and O. Nakano, *J. Antibiotics*, **27**, 1000 (1974).
9) K. Miyazaki, O. Ogino, and T. Arita, *Chem. Pharm. Bull.* (Tokyo), **22**, 1910 (1974).

Buffers——The following buffer systems were employed: pH<2, HCl–KCl; pH 2—8, citric acid–disodium hydrogen phosphate; pH 8—12, disodium hydrogen phosphate–NaOH; pH>12. sodium borate–NaOH. All pH values were measured at the time of use of the buffer solutions.

Effect of pH of the Reaction Media on Production of a Fluorescent Product——One ml of solution containing 40 mcg cephalexin was added to 3 ml of buffers of various pH (pH 3—12) in stoppered test tubes. After heating 1 hr at 100°, pH of these solutions were measured and then buffered to pH 9.5. The volume was adjusted to 10 ml in the volumetric flasks and the fluorescence intensity was subsequently determined.

Rate of Formation of the Fluorescent Product——Five ml of 2 mcg/ml cephalexin solution was added to 15 ml of buffers at pH 12.0 and heated at 100° for 150 min. At 15 min intervals, a 1 ml aliquot was withdrawn and 5 ml of borate buffer at pH 9.5 was added prior to fluorescence measurement.

Effect of Final pH on Fluorescence Intensity——A solution of the fluorescent product was obtained by heating 20 ml of 12.5 mcg/ml cephalexin solution at pH 12.0 for 1 hr at 100°. One ml aliquots of this solution were mixed with 9 ml of each of 13 buffers which covered the pH range of 1—14. The fluorescence intensity and final pH of each solution were then measured.

Solvent Extraction of the Fluorescent Product——Ethyl acetate, chloroform, a mixture of acetone-chloroform, benzene, and ethylene dichloride were used to extract the fluorescent product from aqueous solutions into an organic solvent. The solution containing the fluorescent product formed from cephalexin was adjusted to acidic pH, shaken for 30 min with 7 ml of each organic solvent. The amount of the fluorescent product remaining in aqueous solution was then determined.

Urinary Excretion Experiment——Cephalexin capsules containing 250 mg were given orally with 200 ml of water to 4 healthy volunteers (male; age and body weight of each subject are listed in Table I) following overnight fast. No food was allowed for 4 hr postadministration. Urine was collected every 30 min for 6 hr and every 1 or 2 hr until 10 hr. After measuring volume of urine, small volumes of samples were refrigerated and analysed as soon as practical.

Thin-Layer Chromatography of Cephalexin and Its Possible Metabolite in a Urine Sample——A urine sample after oral dose of cephalexin and the solution of a reference material were spotted on Kieselgel F 254 silica gel sheets. The solvent systems used[10] included ethyl acetate–acetic acid-water (3:1:1) and *n*-butanol–acetic acid–water (3:1:1). A ultraviolet lamp was used to locate the ultraviolet (UV) absorbing substances in the thin-layer sheet.

Results and Discussion

Properties of the Fluorescent Product

An intensively fluorescent product was obtained from cephalexin in alkaline solutions after heating at 100°. The excitation and emission spectra of this product are shown in Fig. 1. All fluorescence measurements in subsequent studies were therefore carried out at the excitation maximum at 355 nm and the emission maximum at 435 nm.

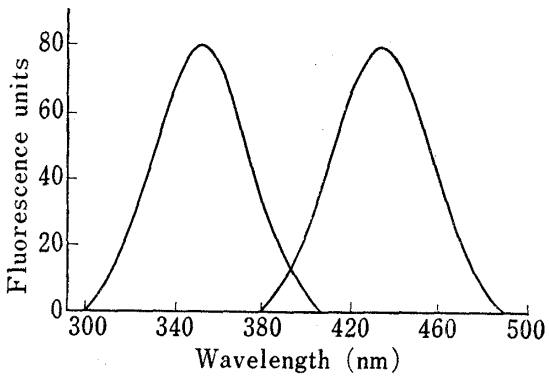

Fig. 1. Spectrophotofluorometric Excitation (355 nm) and Emission (435 nm) Spectra of the Fluorescent Product of Cephalexin

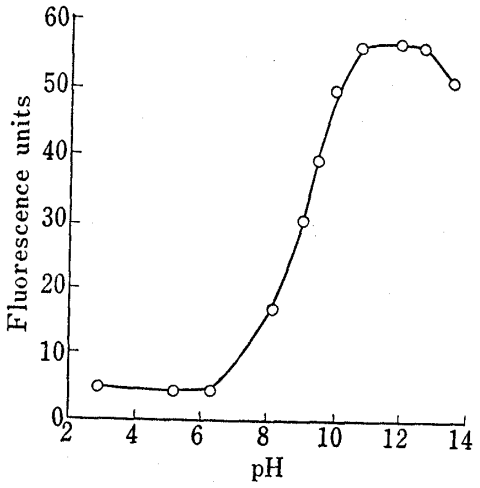

Fig. 2. Effect of pH of the Reaction Media on the Maximum Amount of Fluorescent Product Formed from Cephalexin at 100°

10) M.M. Hoehn and C.T. Pugh, *Appl. Microbiol.*, **16**, 1132 (1968).

The effect of pH of the reaction media on the relative amount of the fluorescent product formed from cephalexin after heating for 1 hr at 100° is shown in Fig. 2. An appreciable quantity of the fluorescent product was generated in alkaline solution with maximum amount formed over the pH range of 11.0—12.5. Yamana, *et al.*[8] showed that cephalexin was stable in acidic solution but was readily hydrolysed in alkaline solution.

The preliminary experiment indicated that elevated temperature was required for formation of the fluorescent product. The rate of formation of the fluorescent product from cephalexin at 100° is shown in Fig. 3. A constant fluorescence intensity was obtained between 45 and 120 min when 0.3M phosphate buffer of pH 12.0 was used.

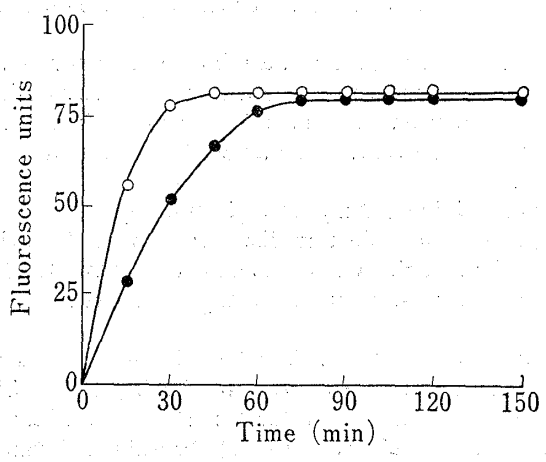

Fig. 3. Rate of Formation of the Fluorescent Product from Cephalexin at 100° in Disodium Hydrogen Phosphate-NaOH Buffers at pH 12

○: 0.3M ●: 0.1M

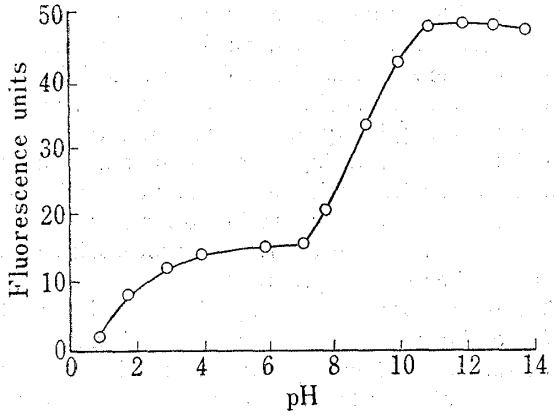

Fig. 4. Effect of pH of the Media at Time of Measurement on Fluorescence Intensity of the Fluorescent Product of Cephalexin

Figure 4 shows the effect of final pH on the fluorescence intensity. The intensity was greater at higher pH values. Under UV irradiation, however, the fluorescence intensity gradually decreased with time at pH higher than 10. The fluorescent product was satisfactorily stable at pH 9.5 as judged by little change in fluorescence intensity for 45 min. It was therefore proposed to adjust the pH of the fluorescent solution to pH 9.5±0.1 prior to the fluorometric determination.

The pH profile of the fluorescent product seems to indicate two ionizable groups (pK_a 1—3 and pK_a 8—10) in the molecule. Possible formation of piperazine-2,5-dione product has been suggested in alkaline hydrolysis of cephalexin.[8,11] Such a product has been identified as an alkaline degradation product of cephradine.[12] Since the proposed product is an amino acid with two ionizable groups, the pH profile is in agreement with the proposed structure of the fluorescent product.

Solvent Extraction

In order to separate the fluorescent product from the interfering materials present in the body fluids, the extractability of the fluorescent product in various pH media into several organic solvents was studied. The product was well extracted into ethyl acetate and the acetone–chloroform mixture, but was practically unextractable into benzene and ethylene dichloride. From the acetone–chloroform (2: 3 v/v) mixture which exhibited the highest extractability, the fluorescent product could be subsequently partitioned back into an alkaline

11) J.M. Indelicato, T.T. Norvilas, R.R. Pfeiffer, W.J. Wheeler, and W.L. Wilham, *J. Med. Chem.*, **17**, 523 (1974).
12) A.I. Cohen, P.T. Funke, and M.S. Puar, *J. Pharm. Sci.*, **62**, 1559 (1973).

solution at pH 9.5. The extractability of the fluorescent product into the acetone-chloroform mixture as the function of pH of the aqueous phase, is shown in Fig. 5. Dependence of extractability on pH cannot be interpreted from the proposed structure of the product. The extraction of the fluorescent product was more efficient at low pH values. It was therefore proposed to make the solution strongly acidic before the extraction procedure.

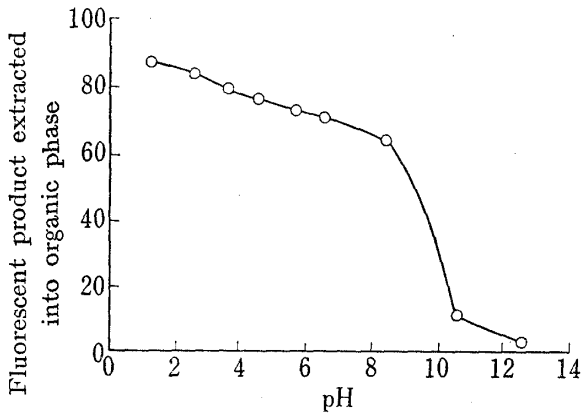

Fig. 5. pH Profile of Extractability of the Fluorescent Product into Acetone–Chloroform (2: 3 v/v) from Aqueous Reaction Mixture

1 ml of urine sample
↓ dilute if necessary
1 ml of sample
↓ add 3 ml of buffer at pH 12.0
heat for 60 min at 100°
add 1 ml of 2 N HCl
add 7 ml of acetone–chloroform (2:3 v/v)
shake for 5 min
centrifuge for 5 min at 2000 rpm
5 ml of organic layer
↓ add 5 ml of borate buffer at pH 9.5
shake for 5 min
centrifuge for 5 min at 2000 rpm
fluorescent measurement of aq. layer
excitation at 355 nm, emission at 435 nm

Chart 1. Proposed Method of Fluorometric Determination of Cephalexin in Urine Sample

Assay Procedure

From the studies described above, the proposed method for the fluorometric assay of cephalexin in urine sample is outlined in Chart 1. According to the procedure, a 1 ml aliquot of sample solution, diluted adequately with distilled water if necessary, was placed in a test-tube. Three ml of the buffer solution at pH 12 (0.3M Na_2HPO_4–NaOH) was added to the sample solution and the mixture was heated for 1 hr at 100° to obtain a solution of the fluorescent product. This solution was cooled to room temperature and 1 ml of 2N HCl was added. To this acidic solution 7 ml of the acetone–chloroform (2: 3 v/v) mixture was added, and the content was vigorously shaken for 5 min and centrifuged. Five ml of the organic layer was then added to 5 ml of 0.1M borate buffer at pH 9.5 (sodium borate–NaOH), and the content was shaken for 5 min and centrifuged. Fluorescence of the aqueous layer was measured.

There was a linear relationship between concentration of cephalexin and fluorometric response in the range of 1—20 mcg/ml urine sample. The standard deviation of this fluorometric assay at 5 mcg/ml of cephalexin in urine sample was $\pm 2.5\%$ ($n=11$).

Other Antibiotics

The assay procedure was tested for possible formation of fluorescent product when cephalexin was substituted for other cephalosporins and ampicillin. Cephaloglycin and ampicillin formed fluorescent products (fluorescence intensity relative to cephalexin were 0.546 ± 0.007 and 0.432 ± 0.005, respectively) whereas cephaloridine and cephalothin did not form any detectable amount of fluorescent product. Antibiotics with α-aminobenzyl group thus formed fluorescent products. It is likely that the aminobenzyl group and a cleaved β-lactam ring are necessary for the formation of the fluorescent product. This is in accord with proposed formation of piperazine-2,5-dione following intramolecular nucleophilic attack by amino group to β-lactam carbonyl.[8]

Urinary Excretion

The excretion rates and cumulative amounts of cephalexin excreted in urine are shown in Table I and Fig. 7, respectively. Cephalexin was well absorbed and rapidly excreted into

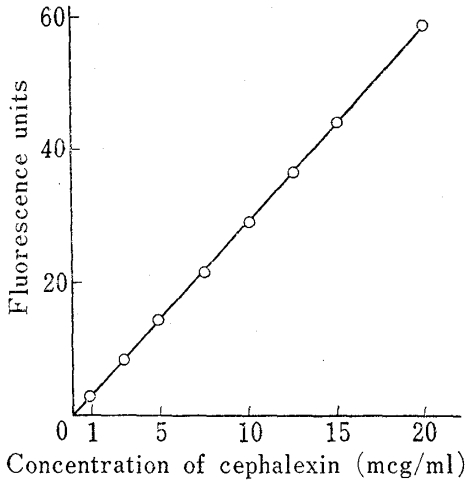

Fig. 6. Fluorometric Standard Curve Obtained for Cephalexin in Urine Sample

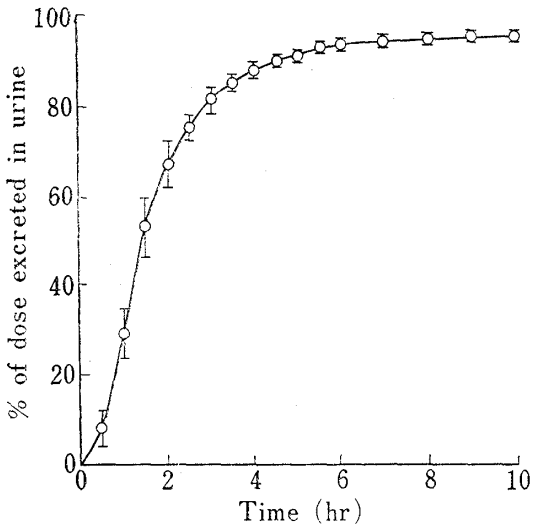

Fig. 7. Cumulative Urinary Excretion of Cephalexin Following Oral Administration of One 250 mg-Capsule to Healthy Male Volunteers

mean and S.E. of 4 experiments

Table I. Urinary Excretion of Cephalexin Following Oral Administration of One 250 mg-Capsule to Healthy Male Volunteers

Subject	A	B	C	D	Mean and S.E.	
Age, years old	23	27	25	25		
Body weight, kg	61	53	60	57		
Time of urine collection, hr	Amount of cephalexin in urine, mg					
0.5	33.7	6.1	0.0	0.0	9.95	8.05
1.0	67.4	68.5	41.5	76.0	63.4	7.53
1.5	41.1	65.3	50.8	74.6	58.0	7.45
2.0	27.2	34.0	45.5	37.6	36.1	3.81
2.5	19.5	20.2	32.0	19.2	22.7	3.10
3.0	12.9	11.4	19.7	6.7	12.7	2.69
3.5	9.7	6.4	14.6	7.6	9.58	1.81
4.0	5.8	4.8	9.9	5.6	6.53	1.15
4.5	5.8	4.1	7.4	2.7	5.00	1.02
5.0	3.3	3.1	5.2	3.0	3.65	0.52
5.5	3.1	2.4	4.4	1.9	2.95	0.54
6.0	1.7	1.4	3.2	1.0	1.83	0.48
7.0	2.9	2.6	3.6	1.5	2.65	0.44
8.0	1.7	1.0	2.3	1.0	1.50	0.31
9.0	0.9	—	1.3	0.0	0.73	0.38
10.0	0.8	0.8	0.6	0.0	0.55	0.19

urine. Urinary recovery of bioactive (unchanged) cephalexin was reported to be 95.7% in 6 hr.[13] This value was in good agreement with our result (93.0% in 6 hr) obtained with the fluorometric assay method.

Little metabolism of cephalexin has been reported both in animals and in humans.[14,15] An attempt was made to find whether any metabolite of cephalexin could be detected by chemical methods. Urine sample was developed on thin-layer chromatographic sheets and

13) P.E. Gower and C.H. Dash, *Brit. J. Pharmacol.*, **37**, 738 (1969).
14) H.R. Sullivan, R.E. Billings, and R.E. McMahon, *J. Antibiotics*, **22**, 195 (1969).
15) M. Gasha, H. Kume, K. Furuta, and S. Sato, *Oyo Yakuri*, **3**, 205 (1969).

the chromatogram was examined under ultraviolet lamp, or dipped in ninhydrin. With each of solvent systems, only one strong spot was obtained from the urine sample collected after oral administration of cephalexin, except for few weak spots of urine blank. In ethyl acetate–acetic acid–water system, *Rf* value of the main spot in urine sample (0.503) was in good agreement with the value of standard solution of cephalexin (0.508). The main spot and other few spots considered to be due to a urine blank was then scraped off the sheet and determined fluorometrically. Only the main spot formed fluorescent product and none of other spots formed any detectable fluorescent product. The similar results were obtained with *n*-butanol–acetic acid–water system It seems most likely that cephalexin is not metabolized to any detectable extent in man and excreted in urine in an unchanged form.

The present assay procedure may be used with some modifications for determination of cephalexin in serum samples.