

Fluorometric Determination of Cephalexin, Cephadrine, and Cephatrizine in Biological Fluids¹⁾

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Intensely fluorescent products were formed from cephalexin and cephradine in acidic solution containing hydrogen peroxide by hydrolysis at high temperature. A fluorescent product was formed from cephatrizine in solutions containing mercuric chloride by warming at 60° for 50 min after cleavage of the β -lactam ring with 1 N NaOH. Each of the fluorescent products was readily extracted with ethyl acetate or an acetone-chloroform mixture from neutral solutions, and could be partitioned into alkaline solution. The urinary excretion of these antibiotics in human volunteers after oral administration was investigated using these new methods.

Keywords—fluorometric determination; biological fluids; cephalexin; cephradine; cephatrizine; urinary excretion

Several fluorometric assay methods for the determination of cephalosporins such as cephalexin,^{3,4)} cephradine,⁵⁾ and several cephalosporins⁶⁾ have been reported in recent years. However, there is no rapid fluorometric method for the common assay of cephalexin and cephradine in biological samples. In addition, there are few reports on sensitive spectrophotometric assay methods for cephatrizine.

In the earlier studies dealing with methods⁷⁾ for the quantitative determination of ampicillin derivatives (ampicillin,^{7a)} pivampicillin,^{7b)} amoxicillin,^{7c)} and talampicillin⁸⁾), it was shown that a fluorescent product could be formed reproducibly by warming sample solutions containing mercuric chloride at 40–50° after hydrolysis of the β -lactam ring by alkali treatment at room temperature. In a preliminary experiment, it was found that the fluorescent product could be formed from cephatrizine by the procedure used for the determination of amoxicillin.^{7c)} However, the fluorescent products from cephalexin and cephradine were not formed quantitatively by the procedure used for ampicillin derivatives,⁷⁾ probably because β -lactam cleavage of these two cephalosporins did not take place quantitatively of alkali treatment at room temperature.

On the basis of these observations, we sought to develop sensitive and reproducible assay methods for cephalexin, cephradine, and cephatrizine in aqueous solutions, urine, and plasma. Using the newly developed assay methods, urinary excretion of these cephalosporins following oral administration was investigated.

- 1) This work was presented at the 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1976.
- 2) Location: *Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo, 060, Japan.*
- 3) R. Aikawa, M. Nakano, and T. Arita, *Chem. Pharm. Bull.* (Tokyo), **24**, 2350 (1976).
- 4) R.H. Barbhuiya and P. Turner, *J. Pharm. Pharmacol.*, **28**, 791 (1976).
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- 7) a) K. Miyazaki, O. Ogino, and T. Arita, *Chem. Pharm. Bull.* (Tokyo), **22**, 1910 (1974); b) K. Miyazaki, O. Ogino, M. Nakano, and T. Arita, *ibid.*, **23**, 178 (1975); c) K. Miyazaki, O. Ogino, H. Sato, M. Nakano, and T. Arita, *ibid.*, **25**, 253 (1977).
- 8) Unpublished data; the fluorometric assay method developed for the determination of ampicillin^{7a)} was applicable for the determination of talampicillin.

Experimental

Materials and Reagents—Cephalexin monohydrate (potency: 925 $\mu\text{g}/\text{mg}$), cephadrine dihydrate (potency: 950 $\mu\text{g}/\text{mg}$), and the anhydrous and mono propylene glycol-solvated forms of cephatrizine (potency: 834 $\mu\text{g}/\text{mg}$) were kindly supplied by Shionogi and Co., Osaka, Japan, Sankyo Co., Tokyo, Japan, and Banyu pharmaceutical Co., Tokyo, Japan, respectively. All the reagents used in the experiments were of reagent grade, and solutions were freshly prepared with redistilled water. As a standard fluorescence solution, a stock solution containing 10 $\mu\text{g}/\text{ml}$ of quinine sulfate was prepared in 0.1 N sulfuric acid, and this solution was diluted appropriately with 0.1 N sulfuric acid before use.

Apparatus—Fluorescence intensity was measured with a Hitachi 203 spectrofluorometer equipped with a xenon lamp.

Buffers—The following buffer systems were employed: pH 2–5, citric acid–HCl–NaOH; pH 5–8, disodium hydrogen phosphate–NaOH; pH 8–9, sodium borate–HCl; pH 9, sodium borate–NaOH. Buffer solutions of pH 2.0, 2.5, and 11.0 were prepared as follows for the determination of cephalexin and cephadrine. Citric acid (21.0 g) was dissolved in 200 ml of 1 N NaOH and the mixture was diluted to 1 l with redistilled water (1/10 M disodium hydrogen citrate solution), then this solution was mixed with 1/10 N HCl to adjust the solution to pH 2.0 or 2.5. Buffer solutions of pH 11.0 were prepared with 1/10 M sodium borate solution and 1/10 N NaOH solution.

Buffer solutions for the determination of cephatrizine were prepared following the previously reported procedure for the determination of amoxicillin.^{7c)}

Assay Procedures for Cephalexin and Cephadrine—Method for Aqueous Solutions and Urine Samples: Using the procedure shown in Chart 1, a 1 ml aliquot of sample solution, adequately diluted with redistilled water if necessary, was placed in a test tube. Four ml of buffer solution of pH 2.0 (for cephalexin) or 2.5 (for cephadrine) was added to the sample solution, then 1 ml of 0.5% (w/v) hydrogen peroxide solution prepared in the same buffer solution (pH 2.0 or 2.5) was added. The mixture was heated at 100° for 70 min (cephalexin) or 55 min (cephadrine) to obtain a solution of the fluorescent product. This solution was cooled to room temperature and the fluorescence intensity was measured with excitation at 340 nm and emission at 420 nm.

In the case of urine sample solutions, 1 ml of 1/2 M disodium hydrogen phosphate solution was added to this fluorescent solution to adjust the medium to pH 5.8. Next, 7 ml of acetone–chloroform (2:3, v/v) mixture was added, and the solution was vigorously shaken for 5 min, then centrifuged. Five ml of the organic layer was added to 6 ml of borate buffer solution, pH 11.0, and the solution was shaken for 5 min then centrifuged. The fluorescence intensity of the aqueous layer was measured as mentioned above.

Method for Plasma Samples Containing Cephalexin: Using the procedure shown in Chart 2, 0.4 ml of plasma sample was added to 4 ml of redistilled water in a 10 ml glass-stoppered centrifuge tube. Three ml of 10% trichloroacetic acid (TCA) solution was then added to this diluted plasma sample and the mixture was centrifuged to obtain a clear supernatant. Three ml of the supernatant, adequately diluted with TCA solution (10% TCA: redistilled water = 3:4) if necessary, was pipetted into a test tube containing 2 ml of 1/10 M disodium hydrogen citrate solution. One ml of 0.5% (w/v) hydrogen peroxide in 1/10 M disodium hydrogen citrate solution was then added to this medium and the mixture (final pH 2.0 \pm 0.2) was heated at 100° for 70 min. This solution was cooled to room temperature and 2 ml of 1/2 M disodium hydrogen phosphate solution was added. Seven ml of the acetone–chloroform (2:3, v/v) mixture was added and the

1 ml of sample (dilute if necessary)

add 4 ml of citrate buffer (pH 2.0 or pH 2.5^{a)}
 add 1 ml of 0.5% hydrogen peroxide in citrate buffer (pH 2.0 or pH 2.5^{a)}
 heat at 100° for 70 min (55 min^{a)}
 cool to room temperature (and measure the fluorescence intensity)^{b)}
 add 1 ml of 1/2 M disodium hydrogen phosphate solution
 add 7 ml of acetone–chloroform mixture (2:3, v/v)
 shake for 5 min
 centrifuge at 2000 rpm for 5 min

5 ml of organic layer

add 6 ml of borate buffer at pH 11.0
 shake for 5 min
 centrifuge at 2000 rpm for 5 min

fluorescence measurement of alkaline solution
 (340 nm excitation and 420 nm emission)

Chart 1. Procedure for the Fluorometric Assay of Cephalexin and Cephadrine in Aqueous Solutions and Urine Samples

- a) Procedure for assay of cephadrine.
 b) Procedure in aqueous solutions.

solution was vigorously shaken for 5 min then centrifuged. Five ml of the organic layer was added to 6 ml of borate buffer solution of pH 11.0, and the solution was shaken for 5 min then centrifuged.

The fluorescence intensity of the aqueous layer was measured as mentioned above.

Method for Plasma Samples Containing Cephadrine: Using the procedure shown in Chart 2, 3 ml of the centrifuged supernatant solution, adequately diluted with TCA solution if necessary, was pipetted into a test tube containing 3 ml of 1/10 M disodium hydrogen citrate solution. Next, 1 ml of 0.6% (w/v) hydrogen peroxide solution prepared in 1/10 M disodium hydrogen citrate solution was added, and the mixture (final pH 2.5 ± 0.2) was heated at 100° for 55 min. This solution was cooled to room temperature and 1 ml of prewarmed 1 M disodium hydrogen phosphate solution was added to this solution. The medium was then subjected to the extraction procedure for the determination of cephalixin mentioned above.

Assay Procedure for Cephatrizine in Aqueous Solutions, Urine, and Plasma Samples: In the measurement of cephatrizine (Chart 3 and 4), some procedures of the method used for amoxicillin,^{7c)} *i.e.*, standing time after adding 1 N NaOH, heating temperature, and heating time, were modified. However, the assay

0.4 ml of plasma sample

add 4 ml of redistilled water
add 3 ml of 10% trichloroacetic acid
centrifuge at 3000 rpm for 10 min

3 ml of supernatant

add 2 ml (3 ml)^{a)} of 1/10 M disodium hydrogen citrate solution
add 1 ml of 0.5% (0.6%)^{a)} w/v, hydrogen peroxide in 1/10 M disodium hydrogen citrate solution
heat at 100° for 70 min (55 min^{a)})
cool to room temperature
add 2 ml of 1/2 M disodium hydrogen phosphate solution
(add 1 ml of 1 M disodium hydrogen phosphate solution)^{a)}
add 7 ml of acetone-chloroform mixture (2:3, v/v)
shake for 5 min
centrifuge at 2000 rpm for 5 min

5 ml of organic layer

add 6 ml of borate buffer at pH 11.0
shake for 5 min
centrifuge at 2000 rpm for 5 min

fluorescence measurement of alkaline solution
(340 nm excitation and 420 nm emission)

Chart 2. Procedure for the Fluorometric Assay of Cephalixin and Cephadrine in Plasma Samples

a) Procedure for assay of cephradrine.

1 ml of sample (dilute if necessary)

add 0.5 ml of 1 N NaOH
allow to stand for 15 min
add 0.5 ml of 1 N HCl
add 4 ml of 0.001% (w/v) mercuric chloride solution
in phosphate buffer at pH 6.0
heat at 60° for 50 min
cool to room temperature

a)

add 1.5 ml of 1 M sodium carbonate solution at time of measurement

add 6 ml of ethyl acetate saturated with redistilled water
shake for 5 min
centrifuge at 2000 rpm for 5 min

5 ml of organic layer

add 6 ml of 1/10 M sodium borate solution
shake for 5 min
centrifuge at 2000 rpm for 5 min

fluorescence measurement of alkaline solution
(345 nm excitation and 420 nm emission)

Chart 3. Procedure for the Fluorometric Assay of Cephatrizine in Aqueous Solutions and Urine Samples

b) Procedure for aqueous solutions.

procedure largely followed that for the determination of total amoxicillin.^{7c)} Fluorescence intensity was measured with excitation at 345 nm and emission at 420 nm.

Urinary Excretion Studies—Cephalexin, cephradine, and cephatrizine capsules containing 125 mg were given orally with 100 ml of water to two adult human males following overnight fasting, and urine samples were collected at suitable intervals. The subjects were 33 and 30 years old, and weighed 58, 50 kg, respectively. The administration schedule followed a crossover design, allowing at least a 3 day interval between tests. No food was allowed for 5 hr after administration.

0.4 ml of plasma sample

add 4 ml of redistilled water
add 3 ml of 10% trichloroacetic acid
centrifuge at 3000 rpm for 10 min

3 ml of supernatant

add 0.5 ml of 2N NaOH
allow to stand for 15 min
add 0.5 ml of 2N HCl
add 2 ml of 0.002% (w/v) mercuric chloride solution in 1/2 M disodium
hydrogen phosphate solution
heat at 60° for 50 min
cool to room temperature
follow the procedure for the determination in urine samples (Chart 3)

fluorescence measurement of alkaline solution
(345 nm excitation and 420 nm emission)

Chart 4. Procedure for the Fluorometric Assay of Cephatrizine in
Plasma Samples

Results and Discussion

Formation and Properties of Fluorescent Products from Cephalexin and Cephradine

In preliminary experiments, it was observed that the fluorescent products could be formed from cephalexin and cephradine in acidic media at high temperature, and that the fluorescence intensity gradually increased with time of heating. Jusko⁹⁾ reported that the formation of the fluorescent product from ampicillin was accelerated by the addition of formaldehyde. Barbhaiya and Turner⁴⁾ also used formaldehyde in a procedure for the determination of cephalexin. However, the amount of the fluorescent product formed from cephradine increased with time in acidic media containing formaldehyde even at 100°, and a constant fluorescence intensity could not be obtained during the experimental period (0—180 min). We therefore examined the effects of oxidizing reagents such as hydrogen peroxide, sodium nitrite, sodium metaperiodate, and potassium chlorate of the formation of the fluorescent products from cephalexin and cephradine in acidic media at high temperature. It was found that an intensely and reproducibly fluorescent product could be formed at 100° in acidic media containing hydrogen peroxide. Therefore, the effects of pH, concentration of hydrogen peroxide, and heating time on the relative amount of fluorescent products formed from cephalexin and cephradine were next studied.

In order to investigate the effect of the pH of the hydrogen peroxide solution of the formation of fluorescent products, reaction mixtures of various pHs (pH 1.5—4) were heated at 100° according to the procedures for the measurement of cephalexin and cephradine in aqueous solutions (Chart 1), and after cooling, the fluorescence intensities were measured at pH 11.0. As shown in Fig. 1, a relatively constant fluorescence intensity was obtained over the pH range of 1.5—2.2 for cephalexin and over the pH range of 2.2—3.0 for cephradine. The pH values of sample solutions of cephalexin and cephradine were therefore adjusted to 2.0 ± 0.2 and 2.5 ± 0.2 , respectively, prior to heating at 100°.

9) W.J. Jusko, *J. Pharm. Sci.*, **60**, 728 (1971).

Figures 2 and 3 show the effects of the concentration of hydrogen peroxide and the heating time of the rate of formation of the fluorescent products. Cephalexin (Fig. 2) and cephradine (Fig. 3) were heated at 100° in media containing various concentrations of hydrogen peroxide. As shown in Fig. 3, in solutions with or without relatively low concentrations of hydrogen peroxide (0.04%, 0.1%), the amount of the fluorescent product formed from cephradine increased gradually with time. On the other hand, a relatively constant fluorescence intensity was obtained after heating for 50 min in media containing 1 ml of 0.5% (w/v) hydrogen peroxide. In the case of cephalexin, a relatively constant fluorescence intensity was also obtained after heating for 60 min in media containing 1 ml of 0.5% (w/v) hydrogen peroxide (Fig. 2). Consequently, in the determination of cephalexin, the fluorescent product was formed by heating the sample solutions at 100° at pH 2.0 for 70 min. Similarly, in the determination of cephradine, the reaction was carried out by heating at 100° at pH 2.5 for 55 min.

In order to investigate the effect of final pH on the fluorescence intensity, 1 ml of a solution containing the fluorescent product formed from cephalexin was mixed with 15 ml each of 12 kinds of buffers covering the pH range of 2–12, and the fluorescence intensity and final pH of each solution were then measured. As shown in Fig. 4, the intensity was greater at higher pH values. A similar pattern was also obtained in the experiments on cephradine. Thus, measurements of the fluorescence intensity in urine and plasma sample solutions were carried out in alkaline solution to increase the sensitivity.

In another experiment, it was found that the fluorescent product could be formed quantitatively from the β -lactam ring cleavage product of cephalexin by warming at 40° for 30 min in the presence of mercuric chloride. Thus, the assay procedure^{7a)} for aminobenzylpeni-

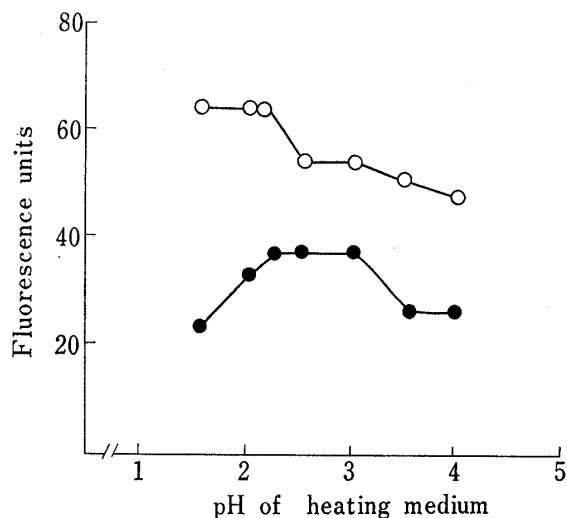


Fig. 1. Effect of pH of the Reaction Medium on the Maximum Amount of Fluorescent Products formed from Cephalexin and Cephradine

○: cephalexin, ●: cephradine.
Fluorescence intensity was measured at pH 11.0.
Concentration of drugs: 10 μ g/ml.

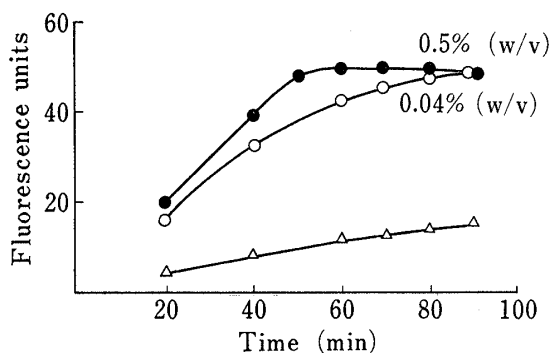


Fig. 2. Rate of Formation of the Fluorescent Product from Cephalexin at 100° in the Presence and Absence of Hydrogen Peroxide

△: without hydrogen peroxide.
Concentration of drug: 5 μ g/ml.

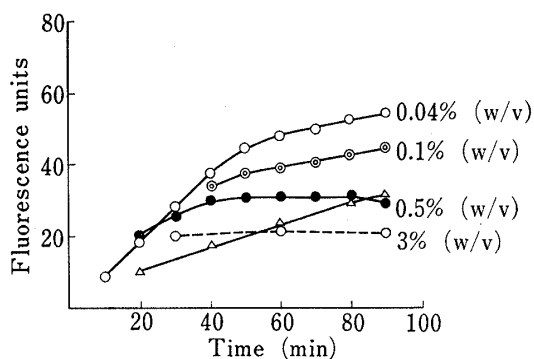


Fig. 3. Rate of Formation of the Fluorescent Product from Cephradine at 100° in the Presence and Absence of Hydrogen Peroxide

△: without hydrogen peroxide.
Concentration of drug: 5 μ g/ml.

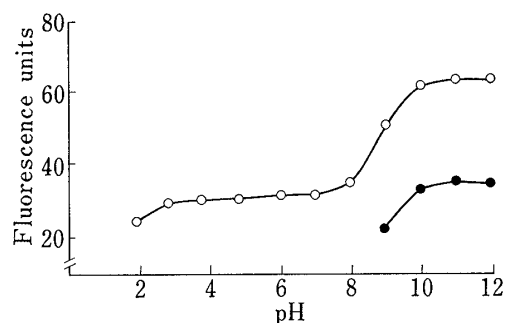


Fig. 4. Effect of pH of the Medium at the Time of Measurement on the Fluorescence Intensity of the Fluorescent Products from Cephalexin and Cephadrine

○: cephalexin, ●: cephradine.
Concentration of drugs: 5 $\mu\text{g/ml}$.

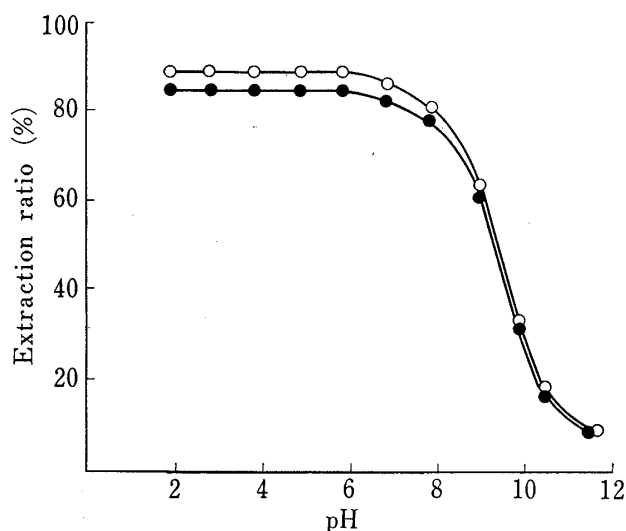


Fig. 5. pH Profiles of Extractability of the Fluorescent Products from Cephalexin and Cephadrine into Acetone-Chloroform Mixture (2:3, v/v) from Aqueous Reaction Mixtures

○: cephalexin, ●: cephradine.
Concentration of drugs: 10 $\mu\text{g/ml}$.

cilloic acid is also applicable for measurement of the β -lactam ring cleavage product of cephalexin.

Solvent Extraction

In order to separate the fluorescent product from interfering materials present in the body fluids, the extractability of the fluorescent product in media at various pH with several organic solvents was studied. The fluorescent product was effectively extracted by ethyl acetate and an acetone-chloroform mixture (2:3, v/v), and the fluorescent product could subsequently be partitioned into an alkaline solution. The extractability of the fluorescent products formed from cephalexin and cephradine by acetone-chloroform mixture is shown in Fig. 5 as a function of the pH of the aqueous phase. Extraction of the fluorescent product was more efficient over the pH range of 2–7.

In the determination of biological samples, however, it was found that unidentified interfering materials were also extracted from the acidic media in organic solvents. This fluorescence owing to interfering materials was reduced to approximately the same level as that of the reagent blank when the extraction was carried out with acetone-chloroform mixture (2:3, v/v) in neutral media. Thus, the pH of the sample solution was adjusted to 5.5–6.5 before extraction.

Over the range of 0.4–4 $\mu\text{g}/0.4\text{ ml}$ of plasma sample, there was a linear relationship between antibiotic concentrations and fluorometer responses, as shown in Fig. 6. The relative recoveries of cephalexin and cephradine from rat plasma samples (2.0 $\mu\text{g}/0.4\text{ ml}$ plasma) were $98.6 \pm 2.2\%$ ($n=10$) and $102.1 \pm 2.1\%$ ($n=10$), respectively. The coefficients of variation of the fluorescence intensities ($n=10$) of cephalexin and cephradine in human urine samples (5.0 $\mu\text{g/ml}$) were 1.0% and 0.9%, respectively. In the case of rat plasma samples (2.0 $\mu\text{g}/0.4\text{ ml}$ plasma, $n=10$), the values of the variance of cephalexin and cephradine were 2.9% and 2.0%, respectively.

Formation and Properties of the Fluorescent Product from Cephatrizine

A preliminary experiment indicated that the properties of the fluorescent product formed from cephatrizine (*e.g.*, extractability and fluorescence spectrum) are similar to those of the fluorescent product formed from amoxicillin.^{7c)} However, the rates of cleavage of the β -lactam

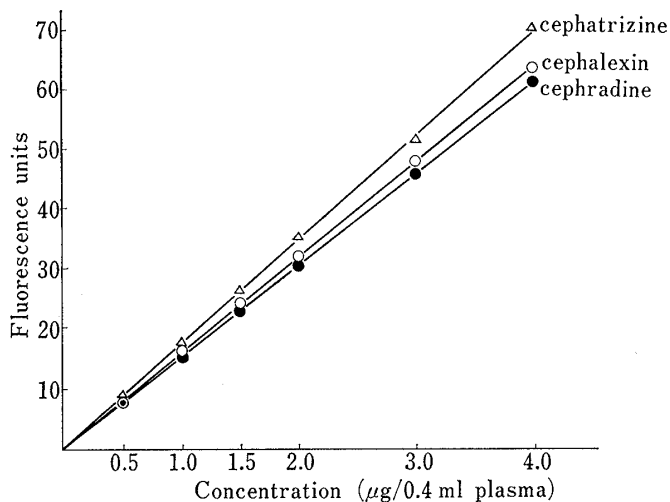


Fig. 6. Fluorometric Standard Curves obtained for Cephalexin, Cephadrine, and Cephatrizine added to Pooled Rat Plasma

Concentrations of standard quinine sulfate solutions: cephalexin (0.1 µg/ml), cephradine (0.04 µg/ml), and cephatrizine (0.04 µg/ml).

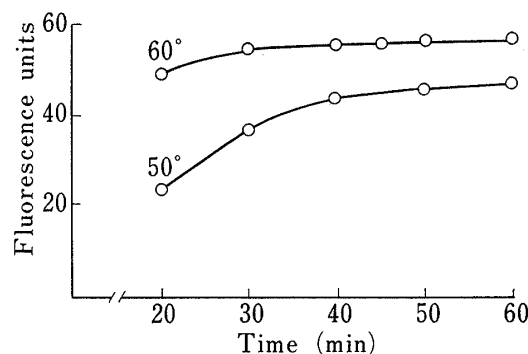


Fig. 7. Relationship between Fluorescence Intensity of the Fluorescent Product from Cephatrizine and Warming Time at 50° or 60°

Concentration of drug: 5 µg/ml.

ring of cephatrizine in alkaline solution and of formation of the fluorescent product were slightly slower than those of amoxicillin. Figure 7 shows the effects of temperature and heating time on the rate of formation of the fluorescent product from cephatrizine according to the procedure shown in Chart 3. A maximum and constant fluorescent intensity was obtained between 50 and 60 min on heating at 60°. Other conditions for the formation of the fluorescent product, *i.e.*, the concentration and pH of the mercuric chloride solution, the effect of pH of the media during measurement, and the extractability with organic solvent, were similar to those used in the determination procedure for amoxicillin.^{7c)}

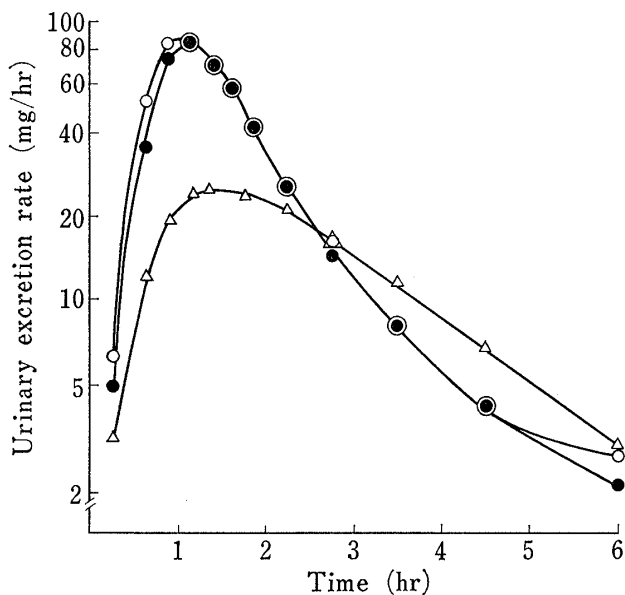


Fig. 8. Urinary Excretion Rates of Cephalexin, Cephadrine and Cephatrizine following Oral Administration (125 mg Capsule) to Two Healthy Male Volunteers

Data are means of two experiments.
○: cephalexin, ●: cephradine, △: cephatrizine.

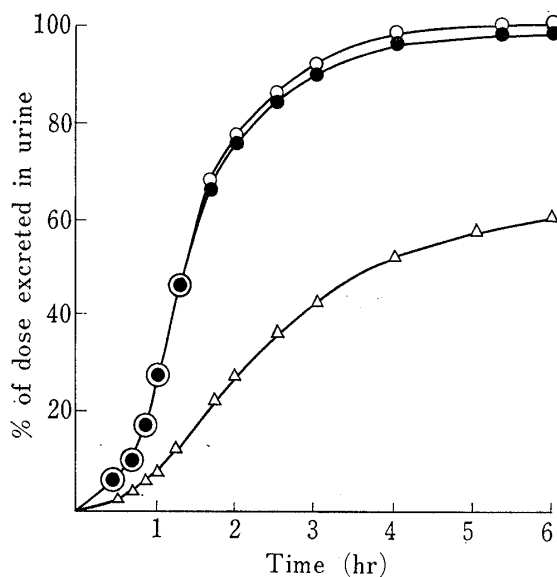


Fig. 9. Cumulative Urinary Excretion of Cephalexin, Cephadrine, and Cephatrizine following Oral Administration (125 mg Capsule) to Two Healthy Male Volunteers

Data are means of two experiments.
○: cephalexin, ●: cephradine, △: cephatrizine.

Over the range of 0.4—4 $\mu\text{g}/0.4$ ml of plasma sample, there was a linear relationship between cephatrizine concentration and fluorometer response, as shown in Fig. 6. The relative recovery of the antibiotic from rat plasma samples (2.0 $\mu\text{g}/0.4$ ml plasma) was $97 \pm 2.1\%$ ($n=10$). The coefficient of variation of the fluorescence intensity ($n=10$) of the antibiotic in human urine samples (5.0 $\mu\text{g}/\text{ml}$) was 2.8%. On the basis of these results, the proposed methods for the fluorometric assay of cephatrizine in aqueous, urine, and plasma samples are outlined in Charts 3 and 4.

Urinary Excretion

The excretion rates and cumulative amounts of the antibiotics in urine are shown in Fig. 8 and 9, respectively. Cephalexin and cephradine were quickly absorbed and rapidly excreted in urine, while the urinary recovery of cephatrizine in 6 hr was only 57% of the dose. Studies on the intestinal absorption of these amphoteric β -lactam antibiotics may therefore be of interest.

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