THE FATE OF PARENTERAL CEPHALOTHIN AND RELATED ANTIBIOTICS IN THE RAT

THE NATURE OF A MINOR LONG-LIVED METABOLITE

HUGH R. SULLIVAN, ROBERT E. MCMAHON
and RICHARD E. HEINEY

The Lilly Research Laboratories, Eli Lilly and Company,
Indianapolis, Indiana 46206, U.S.A.

(Received for publication March 8, 1971)

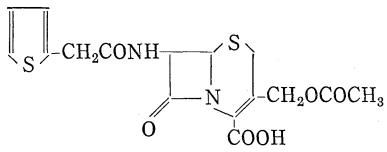
Intraperitoneally administered cephalothin-^{14}C is rapidly metabolized and excreted in the rat with about 70 % of a radioactive dose appearing in urine in 4 hours. The major metabolite is desacetylcephalothin which forms by hydrolysis of the ester group. Some *in vivo* side chain hydrolysis at position 7 also occurs as indicated by the excretion of thienylacetylglycine, a metabolite of thiophene acetic acid. A long-lived minor metabolite occurs in blood and appears to be an albumin-cephalothin complex. A similar complex forms from cephaloglycin, 7-phenylacetamidocephalosporanic acid and 6-thienylacetamidopenicillanic acid but not from desacetylcephalothin, cephalexin, penicillin G or penicillin V. The complex was formed *in vitro* by incubation of radiocephalothin with rat serum.

It should be noted that desacetylcephalothin is an antimicrobial agent in its own right and for this reason plasma and urine levels measured by microbiological assays represent the summation of cephalothin and desacetylcephalothin levels. Deacetylation has been shown to be the major route of metabolism of a number of other 7-acylaminocephalosporanic acids including cephaloglycin[4], 7-phenylacetamidocephalosporanic acid[5] and 7-(3-chlorophenyl)-acetamidocephalosporanic acid[6]. Cephalexin which has a methyl group instead of the acetoxymethyl group is excreted unchanged thus demonstrating the stability of the cephalosporin nucleus in the mammal[7].

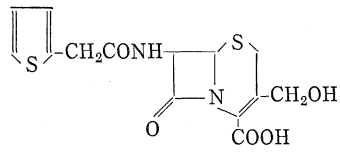
When given orally the two major urine metabolites of cephalothin are thienylacetylglycine and thienylacetamidoethanol[8]. Neither of these substances possess antibiotic activity. One of the purposes of the labeled study described below was to determine the nature of the metabolites of cephalothin other than those detectable by microbiological assay.

Cephalothin, 7-(2-thienylacetamido)-cephalosporanic acid, is an effective broad-spectrum antibiotic when administered parenterally[1]. It is active against gram-positive cocci, including penicillin-resistant strains, as well as against many gram-negative bacilli[2]. The major route of metabolism of cephalothin following parenteral administration to dogs, rats and humans has been shown to be hydrolysis to desacetyl cephalothin[3].

A second purpose of this study was to investigate the possible formation of a



Cephalothin



Desacetylcephalothin

long-lived plasma metabolite such as that indicated by the previous studies with radiocephaloglycin[4]. The radiocarbon tracer studies described below demonstrated that such a long-lived metabolite of cephalothin is formed in the rats. The data presented indicate that the metabolite is an albumin-cephalothin complex.

Materials and Methods

Labeled Antibiotics. The preparation of cephalothin-^{14}C[8], 7-phenoxy-1-^{14}C-acetamidocephalosporanic acid[8], cephalexin-^{14}C[7] and 7-phenyl-1-^{14}C-acetamidocephalosporanic acid[5] has been described. In the case of 7-phenyl-1-^{14}C-acetamidocephalosporanic acid, a modified procedure was used to insure that the product would be free of the desacetyl impurity observed in the earlier preparation. Desacetoxycephalothin-^{14}C was prepared by acylation of 7-aminodesacetoxycephalosporanic acid with the acid chloride of thienylacetic acid-1-^{14}C by the procedure of RYAN, SIMON and VAN HEYNINGEN[9]. Desacetylcephalothin-^{14}C was prepared for us by Mr. D. FUKUDA and Dr. D. BRANNON by enzymatic hydrolysis of cephalothin-^{14}C. Radiopenicillin V (6-phenoxy-1-^{14}C-acetamidopenicillanic acid) was supplied by Dr. F. J. MARSHALL. Radiopenicillin G (6-phenyl-1-^{14}C-acetamidopenicillanic acid) was purchased from Amersham-Searle, Arlington Heights, Illinois. The preparation of 6-thienyl-1-^{14}C-acetamidopenicillanic acid was accomplished by the acylation of 6-aminopenicillanic acid with the acid chloride of thienylacetic acid-1-^{14}C.

All of the labeled antibiotics were prepared and stored as potassium salts. Chromatography studies showed these salts to be free of impurities detectable by either radioactive or microbiological assay. For animal studies freshly prepared aqueous solutions (4.6 μmoles/ml) were used.

Chromatography. The paper chromatography system described by MILLER[10] was used. Microbiological zones were located by bioautography on agar plates with *B. subtilis* while radioactive zones were located by scanning, and quantitated by liquid scintillation counting. Methyl esters of thiophene acetic acid and its glycine conjugate were separated on silica gel plates with a 10 : 1 EtOAc - PhH system. Combination gas chromatography-mass spectroscopy was accomplished with an LKB 9000 instrument. A 1 % W-98 column at 150° was used for separation.

Excretion Studies. Radiocephalothin was administered intraperitoneally to 200-g male Purdue-Wistar rats at a dose rate of 46 μmoles/kg body weight (20 mg/kg). The rats were kept in stainless steel metabolism cages. Urine samples were collected periodically and the radiocarbon content determined by liquid scintillation counting. The nature of the metabolites present in pooled urine samples was determined by paper chromatography as described above.

For the bile study, 200-g rats were anesthetized with ether and a cannula placed into the common bile duct. Following administration of labeled antibiotic, bile was collected for 24 hours and the radiocarbon content determined by liquid scintillation counting.

Blood and Tissue Levels. Labeled antibiotics were administered intraperitoneally to rats at a dose rate of 46 μmoles/kg body weight. At selected time intervals, blood samples were removed from the tail vein with heparinized pipettes. These samples, averaging 200 mg in weight, were proccessed for liquid scintillation counting using the wet tissue digestion method of MAHIN and LOFBERG.[11]

For the tissue level determinations, cephalothin-^{14}C (46 μmoles/kg) was administered orally to 6 male Purdue-Wistar rats and 6 I.C.R. (Cox) mice. One and four hours after administration, a group of three animals was sacrificed by decapitation. The appropriate tissues and organs were removed and their wet weights were recorded. A weighed sample of each tissue and organ was removed and prepared for liquid scintillation counting employing the wet tissue digestion method.

Characterization of Long-Lived Cephalothin Metabolite in Serum. An aqueous solution of [^{14}C]cephalothin was administered intraperitoneally to a group of three 200-g male Purdue-Wistar rats at a dose rate of 46 μmoles/kg. Twenty-four hours after administration, the total available blood was obtained from each rat by cannulation of the orbital sinus vein. The blood from the three rats was pooled (14.0 ml) and allowed to coagulate at 5°C for 2 hours. The serum (7.0 ml) was separated by centrifugation.

Serum (6.5 ml) was then placed in a suitable length of Visking viscose-cellulose dialysis tubing (2.5-cm internal diameter) and dialyzed with stirring against 65.0 ml of veronal buffer solution (pH 8.5) for 72 hours. At this time both the serum and the diffusate were sampled for radiocarbon determination. The dialyzed serum was separated into its component protein fractions; *i.e.*, albumin, α-, β- and γ-globulins; employing both free and gel electrophoresis.

Moving boundary electrophoresis was performed in a Beckman/Spinco Model H electrophoresis-diffusion instrument using a veronal buffer (pH 8.6), $\Gamma/2=0.1$ a 1°. Fractions were separated by standard sampling techniques. Subsequently, an aliquot of each fraction was dissolved in 1.0 ml of Nuclear-Chicago protein solubilizer, 10 ml of Diatol liquid scintillator added and the radiocarbon content determined by liquid scintillation counting.

Acrylamide gel electrophoresis was also utilized employing the conditions and techniques described by DAVIS.[12]. The acrylamide gel containing the resolved serum protein components was subsequently sectioned and dissolved in 30 % hydrogen peroxide[13]. A 1.0 ml portion of Nuclear-Chicago solubilizer was added to insure complete solubility of the protein material and 10 ml of Diatol liquid scintillator added for measurement of the radiocarbon content.

In Vitro Formation of Long-Lived Serum Metabolite. To 4.5 ml of fresh rat serum 9.2 μmoles of radiocephalothin was added in 2 ml of water containing 20 mg of potassium chloride. The solution was incubated at 37°C for 2 hours and then dialyzed against repeated changes of veronal buffer (pH 8.5). After determining the amount of undialyzable remaining radioactivity, the dialyzed serum solution was subjected to protein separation by free electrophoresis. The albumin fraction (1.2 ml) which contained almost all of the radioactivity (97 %) was dialyzed against normal saline to remove residual veronal. One ml of this solution of the labeled cephalothin-albumin complex was administered intravenously to a rat and the rate of disappearance of radioactivity from the blood determined.

Results and Discussion

Excretion Studies

The rate of appearance of radioactive metabolites in rat urine following intraperitoneal administration of radiocephalothin is summarized in Table 1. Excretion into urine was rapid with about 50 % recovery in 2 hours. The rate of recovery in rat correlates well with recovery rates in dogs after intravenous and intramuscular administration[3]. The rates also are similar to those seen in rats with radiocephaloglycin[4].

In a separate group of three rats the extent of excretion into bile was determined. An average of 6.3 % of the radioactive dose was found in bile in the first 24 hours.

Table 1. The rate of appearance of labeled metabolites in the urine of rats following intraperitoneal administration of cephalothin-^{14}C (46 μmoles/kg).

Time (hours)	Accumulated % Ra dose appearing in urine			
	Rat 1	Rat 2	Rat 3	Average
0~2	48.6%	52.4%	70.9%	57.3%
4	68.2	64.3	78.1	70.2
6	70.6	68.2	79.8	72.1
8	70.6	70.2	80.6	73.0
10	82.6	71.2	83.4	78.3
12	82.6	71.9	83.4	79.0
14	86.2	72.4	84.3	80.5
16	88.0	72.8	84.6	81.3
18	88.8	73.3	84.8	81.8
20	89.2	73.6	85.5	82.3
22	89.5	73.7	85.9	82.6
24	89.7	74.1	86.1	82.9

Table 2. Tissue levels of labeled antibiotic plus metabolites (calculated as μg equivalents of cephalothin) in rats and mice after an intraperitoneal dose of cephalothin-^{14}C. Each value represents a mean value from three animals.

Tissue	μg equivalents of cephalothin/g.			
	Rat		Mice	
	1 hour	4 hours	1 hour	4 hours
Blood	10.48	2.49	5.52	3.20
Liver	18.01	4.04	6.66	3.43
Spleen	2.45	1.00	2.02	1.02
Kidney	72.63	12.98	10.68	10.73
Lung	4.48	1.58	2.77	1.48
Heart	2.17	1.04	1.55	0.93
Fat	6.42	2.61	7.59	3.75
Muscle	2.37	0.80	1.39	0.50
Brain	0.46	0.48	0.30	0.16

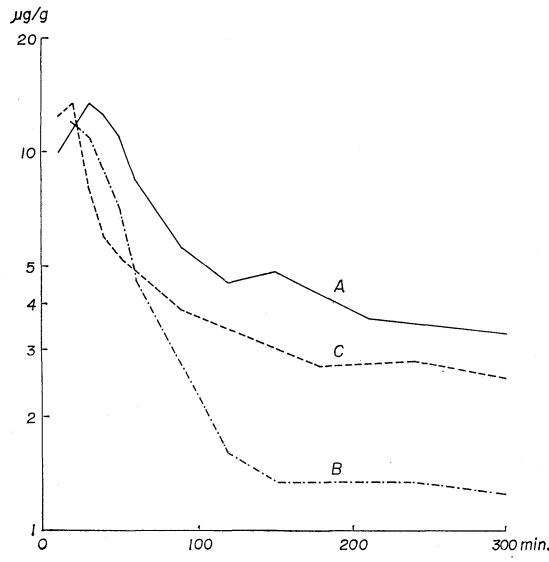
Tissue and Blood Levels

The residues of radiocarbon labeled metabolites in various tissues were determined following intraperitoneal administration of radiocephalothin to rats and to mice. The results are shown in Table 2. As was the case with cephaloglycin[4] the only tissues with higher levels than blood were the tissues involved in metabolism and elimination, liver and kidney. In general, the one hour levels in mice are lower than in rats indicating a more rapid elimination in the mouse. The substantial fall in levels between one hour and four hours in rats was consistent with rapid urinary excretion.

Fig. 1 shows the rate of disappearance of radiocephalothin and metabolites from whole blood. Like cephaloglycin the curve appears to be biphasic. Over the time period 25~100 minutes during which the urinary excretion is maximal, the half-time of disappearance of total radiocarbon was estimated to be 40 minutes. In Fig. 1 blood levels for two analogs of cephalothin are also given. Both disappear at least as rapidly as does cephalothin. The bimodal character of the curves in Fig. 1 suggest the possibility that a long-lived metabolite is present in blood. The nature of this metabolite is discussed in a later section.

Fig. 1. Rate of disappearance of radiocarbon from blood of rats receiving cephalothin-^{14}C (A), 7-phenylacetamido-1-^{14}C-cephalosporanic acid (C) and 7-phenoxyacetamido-1-^{14}C-cephalosporanic acid (B).

Each curve represents a mean value from three rats. The results are calculated as μg equivalents of labeled antibiotics.



The Nature of Urinary Metabolites

The 0~4 hour urines from rats receiving radiocephalothin were combined and the identity of the metabolites present was determined by a combination of chromatographic procedures. An aliquot of the urine was chromatographed on a paper in a water - butanone-2 system. The zones corresponding to cephalothin and desacetylcephalothin were located by bioautography. The amount of each present was then established by counting the same areas on an identical duplicate tape. In this manner it was shown that 63 % of the radioactivity in urine was desacetylcephalothin while only 6 % was present as unchanged cephalothin. Thus the ratio of desacetylcephalothin to cephalothin in urine is greater than 10. Although similar ratios have been reported in dogs it is much greater than that seen in 0~6 hour urines in humans[3].

In addition to the desacetylcephalothin and cephalothin zones, the paper chromatograms had a radioactive zone (16 % of urinary radioactivity) at the origin and a poorly defined zone in front of the desacetylcephalothin. No radioactive zones were seen corresponding to lactone or to thienylacetamidoethanol. In order to determine the nature of the metabolites present in the faster moving, poorly defined zone, an aliquot of urine was extracted with dichloromethane at pH 2. After conversion to methyl esters by treatment with diazomethane the metabolites were separated by thin-layer chromatography. Two metabolites, thiophene acetic acid (0.9 % of urine radioactivity) and thienylacetylglycine (7.6 %), were identified by this procedure. The structure of the latter metabolite was confirmed by combination gas chromatography-mass spectroscopy. The spectrum of the metabolite showed a mass ion of 213 together with fragmentation peaks at 182, 154, 124 and a base peak at 97 ($C_4H_3SCH_2^+$). This spectrum was identical to that seen with the authentic compound.

The presence of thienylacetylglycine, which is the major metabolite of thienylacetic acid, demonstrates that some hydrolysis of cephalothin occurs in the intact animal. It is unlikely that this occurs by excretion into bile followed by hydrolysis in the gut and reabsorption. The oral studies reported earlier[8], which showed that gut metabolism occurred very slowly, discount the possibility that radioactive metabolite would appear in 0~4 hour urines by the enterohepatic route.

Preliminary studies with urine from patients receiving cephalothin have been carried out. It was concluded from these that humans, unlike rats, do not excrete thienylacetylglycine as a metabolite of cephalothin.

The Nature of the Long-Lived Metabolite in Blood

In an earlier study of the fate of cephaloglycin-^{14}C in the rat, evidence for a minor long-lived serum metabolite was found[4]. The nature of the radiocarbon blood level curves shown in Fig. 1 indicate that both labeled cephalothin and 7-phenylacetamidocephalosporanic acid may also form such a metabolite. In order to investigate this matter further, the rate of disappearance of radiocarbon from the blood of rats receiving various labeled cephalosporin derivatives was followed for 70 hours. The results summarized in Fig. 2, confirm that both cephalothin and its phenylacetamido analog form a metabolite that is cleared from blood relatively slowly.

Cephalexin and 7-phenoxyacetamido-cephalosporanic acid, on the other hand, formed such metabolites only in negligible amounts.

The long-lived metabolite could have formed directly from cephalothin or could have been a secondary degradation of desacetylcephalothin, the major metabolite of cephalothin. In order to answer this question the radiocarbon levels in blood were determined following administration of radiodesacetylcephalothin to rats. It is clear from these studies (Fig. 3) that the long-lived metabolite does not arise from desacetylcephalothin and must form directly from cephalothin by some other pathway. In this same study desacetoxycephalothin-^{14}C, the thienylacetamido analog of cephalexin, was also investigated. The result, show in Fig. 3, was very similar to that seen with cephalexin itself (Fig. 2), *i.e.* long-lived metabolite formation was minimal when compared to cephalothin.

Fig. 2. Whole blood radiocarbon levels in rats after intraperitoneal administration of cephalothin-^{14}C (A), 7-phenylacetamido-1-^{14}C-cephalosporanic acid (B), 7-phenoxyacetamido-1-^{14}C-cephalosporanic acid (C), and cephalexin-^{14}C (D).
Each curve is based on three rats.

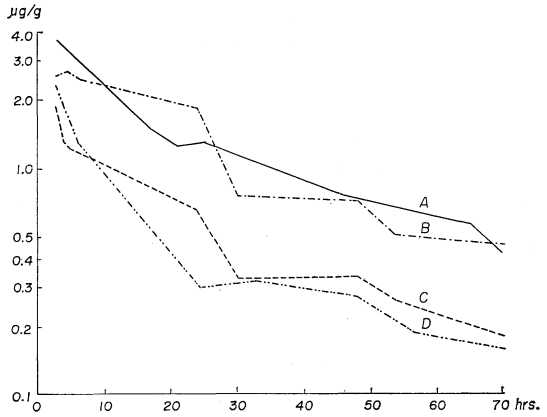
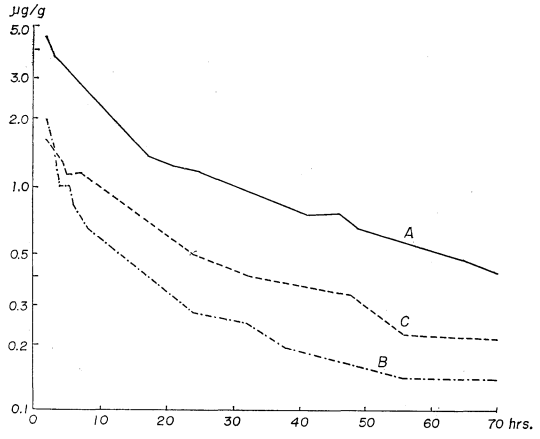


Fig. 3. Whole blood radiocarbon levels in rats after intraperitoneal administration of cephalothin-^{14}C (A), desacetoxycephalothin-^{14}C (B) and desacetylcephalothin (C).
Three rats were used in each experiment.



It seemed of interest to investigate whether or not the penicillin antibiotics formed a similar long-lived metabolite. The whole blood levels of radiocarbon in rats receiving each of three labeled penicillin derivatives were determined. The results (Fig. 4) showed that of the three, only 6-thienylacetamidopenicillanic acid, the penicillin analog of cephalothin, gave appreciable levels of a persistant metabolite.

The nature of the metabolite was further investigated in the following fashion. Blood was obtained from a rat 24 hours following the administration of cephalothin-^{14}C. Serum prepared from this blood had a radiocarbon content equivalent to 1.46 μg (0.003 μmole) cephalothin-^{14}C/ml. From this result the amount of long-lived metabolite present in the serum compartment was estimated to be about 0.4 % of the administered cephalothin.

The observation that less than 5 % of the radioactivity in the serum could be removed by dialysis suggested that the long-lived metabolite might be a conjugate of cephalo-

thin and a serum protein. In support of this suggestion it was found that when the serum proteins were separated by either free electrophoresis or analytical gel electrophoresis over 95 % of the radioactivity was recovered bound to albumin. Radioactivity was not detected in the globulin fractions.

The nature of the long-lived metabolite of cephalothin was confirmed by *in vitro* treatment of rat serum with cephalothin-^{14}C (1.4 μ mole/ml) for 2 hours at 37°C and then dialysis until all free cephalothin had been removed. Under these conditions 0.37 μmole of radiocephalothin was bound per ml of serum solution. Electrophoresis showed that essentially all of this was associated with albumin. A sample of the serum containing the albumin-radiocephalin complex was then administered intravenously to a rat. The rate of disappearance of radiocarbon which was observed (Fig. 5) proved to be in the same range (25~30 hours) as that of the metabolite formed *in vivo*.

Fig. 4. Whole blood radiocarbon levels in rats after intraperitoneal administration of 6-thienylacetamido-1-^{14}C-penicillanic acid (B), penicillin-V-^{14}C (A) and penicillin-G-^{14}C (C).

Each experiment employed three rats.

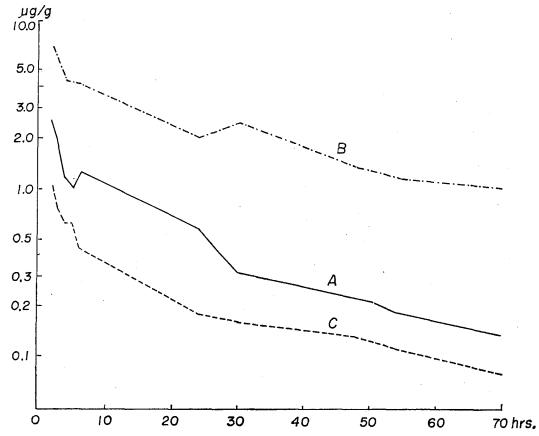
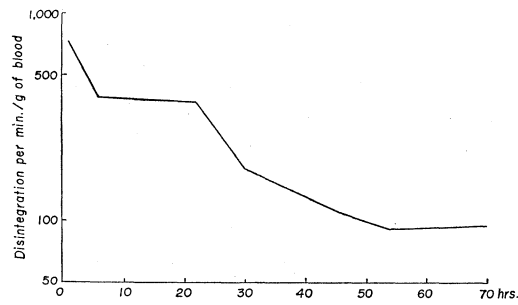


Fig. 5. Whole blood radiocarbon level decay after intravenous administration of preformed albumin-radio cephalothin complex.

Details of the experiment are described in Materials and Methods.



Based on the information now available it is probable that the long-lived metabolite formed from cephalothin and related antibiotics is a complex formed between the antibiotic and albumin. The complex may form by chemical reaction between albumin and antibiotic. However, it is also possible that complex formation results from a physical binding of sufficient strength that it appears to be irreversible. The long half-life of the complex suggests that its breakdown occurs as a consequence of the normal metabolism of the albumin molecule.

References

1) Chauvette, R. R.; E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. B. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer & E. M. Van Heyningen: Chemistry of cephalosporin antibiotics. II. Preparation of a new class of antibiotics and the relation of structure to activity. J. Am. Chem. Soc. 84 : 304~305, 1962

2) Boniece, W. S.; W. E. Wick, D. H. Holmes & C. E. Redman: *In vitro* and *in vivo* laboratory evaluation of cephalothin, a new broad spectrum antibiotic. J. Bact. 84 : 1292～1296, 1962
3) Leo, C. C.; E. B. Herr, Jr. & R. C. Anderson: Pharmacological and toxicological studies on cephalothin. Clin. Med. 70 : 1123～1138, 1963
4) Sullivan, H. R.; R. E. Billings & R. E. McMahon: Metabolism of D-cephaloglycin-^{14}C and L-cephaloglycin-^{14}C in the rat. J. Antibiotics 22 : 27～33, 1969
5) Culp, H. W.; F. J. Marshall & R. E. McMahon: Metabolism and absorption of 7-(phenylacetamido-1-^{14}C)-cephalosporanic acid. Antimicr. Agents & Chemoth. -1963 : 243～246, 1964
6) Okui, M.; K. Hattori & M. Nishida: Studies on the metabolism of cephalosporins. I. The distribution and degradation of 7-[(1-^{14}C)-3-chlorophenyl-acetamido]-cephalosporanic acid. J. Antibiotics 20 : 287～292, 1967
7) Sullivan, H. R.; R. E. Billings & R. E. McMahon: Metabolism of cephalexin-^{14}C in mice and in rats. J. Antibiotics 22 : 195～200, 1969
8) Sullivan, H. R. & R. E. McMahon: Metabolism of oral cephalothin and related cephalosporins in the rat. Biochem. J. 102 : 976～982, 1967
9) Ryan, C. W.; R. L. Simon & E. M. Van Heyningen: Chemistry of cephalosporin antibiotics. XIII. Desacetoxycephalosporins. The synthesis of cephalexin and some analogs. J. Med. Chem. 12 : 310～313, 1969
10) Miller, R. P.: A paper chromatographic assay for cephalosporins. Antibiot. & Chemoth. 12 : 689～693, 1962
11) Mahin, D. T. & R. T. Lofberg: A simplified method of sample preparation for determination of tritium, carbon-14, or sulfur-35 in blood or tissue by liquid scintillation counting. Anal. Biochem. 16 : 500～509, 1966
12) Davis, G. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121, Art. 2 : 404～427, 1964
13) Young, R. W. & H. W. Fulhorst: Recovery of S^{35} radioactivity from protein-bearing polyacrylamide gel. Analyt. Biochem. 11 : 389～391, 1965