PROBLEMS IN THE BIO-ASSAY OF ORALLY ADMINISTERED CEPHALOGLYCIN IN BIOLOGICAL FLUIDS AND METHOD FOR THE DETECTION OF ITS METABOLITE, DESACETYLCEPHALOGLYCIN

KIHACHIRO SHIMIZU

Department of Central Medical Technology, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan

HARUO NISHIMURA

Clinical Technology Department, Shionogi & Co., Ltd., Fukushima-ku, Osaka, Japan

(Received for publication October 22, 1969)

Disagreements in serum and urine cephaloglycin determinations by *Sarcina lutea* with those by *Bacillus subtilis* or *Streptococcus hemolyticus* after oral administration of the antibiotic have been clarified by the present studies, attention being focussed on the formation of an active metabolite, desacetylcephaloglycin. The formation of desacetylcephaloglycin was detected by paper and thin-layer chromatography. Desacetylcephaloglycin was 0.1 to 0.5 as active as cephaloglycin against *B. subtilis* and *S. hemolyticus* but of comparable activity against *S. lutea* PCI-1001.

Cephaloglycin is therapeutically effective in experimental infections in mice by oral treatments[1]. Because of its good absorption, this cephalosporin C antibiotic appears to be a promising antibiotic for oral administration. For determining concentrations of cephaloglycin in blood and urine after oral administration, cylinder-plate method using *Sarcina lutea* PCI-1001 (ATCC 9341) as the test organism has been reported by WICK *et al.*[1] and RONALD *et al.*[2] As a consequence of intensive studies on the use of this organism for the bio-assay of this antibiotic in blood and urine samples, we found that values obtained with *S. lutea* as the test organism were greater than those with *Bacillus subtilis* and *Streptococcus hemolyticus* as the test organisms.

The purpose of this report, therefore, is to present problems in the bio-assay method of cephaloglycin in body fluids, especially as affected by the test organisms and a method for the detection of cephaloglycin and its metabolite, desacetylcephaloglycin, in urine using a thin-layer or a paper chromatographic system.

Materials and Methods

Preparation of antibiotics: Samples of cephaloglycin and desacetylcephaloglycin were supplied by Lilly Research Laboratories, U. S. A. Because of the instability of desacetylcephaloglycin, the compound was stored in a refrigerator. Working standard solutions of both antibiotics were prepared daily by dissolving antibiotics in 0.1 M potassium phosphate buffer, pH 7.0, and then diluted with the buffer to definite concentrations.

Test samples of serum and urine: Serum and urine samples for antibiotic assay were obtained at various intervals after a single oral administration of 500 mg of cephaloglycin to ten healthy volunteers. The test serum and urine were usually diluted to two-fold and 100-fold with the buffer (pH 7.0), respectively.

Cylinder-plate assay with *S. lutea* PCI 1001 and *B. subtilis* PCI 219: A 24-hour brain heart infusion culture of *S. lutea* and a spore suspension of *B. subtilis* which was prepared by the method recommended for Ministry of Health and Welfare of Japanese Government (Minimum Requirements for Antibiotic Products) for the assay of streptomycin by the plate-diffusion method were employed as stock inoculum and stock spore suspension, respectively. Assay plates were prepared by adding 20 ml of brain heart infusion containing 2 % agar at pH 6.4 to each 90 mm × 22 mm Petri dish and after solidification of the base layer, a 4 ml seed layer was added. This layer was prepared by adding 1 % of stock solution of *S. lutea* or 2 % of spore suspension of *B. subtilis* to melted agar at 48°C. After the agar had hardened, four cylinders were placed on the inoculated agar surface. After the plate had been incubated for 24 hours at 37°C, the diameters of zones of inhibition were read. Sample concentrations were calculated from a reference standard curve.

Superposition assay (one-dimensional diffusion assay method) with *S. hemolyticus* D: A 10 ml amount of a 24-hour 5 % rabbit blood broth culture of *S. hemolyticus* was employed as stock inoculum. Brain heart infusion containing 1 % agar at pH 6.4 was used as basal medium. A 100 ml of basal medium at 48°C was added with 8 ml of defibrinated rabbit blood and then seeded with 0.5 ml of 10-fold dilution of the stock inoculum. A 1 ml of this seeded agar was pipetted into 80 mm × 7 mm test tube (height of the seeded agar is about 40 mm). After solidification of the seeded agar, the tubes were stored in a refrigerator at 5°C for 2~5 hours before use. Each 0.5 ml of standard dilutions of antibiotic and test samples were added to the test tube containing the seeded agar and after incubation for 24 hours at 37°C, the lengths of inhibition zones were measured. The lengths of inhibition zones were plotted against the log of the concentrations of the standard.

Paper and thin-layer chromatography: Known concentrations of cephaloglycin or desacetylcephaloglycin and the test samples of urine were chromatographed on Toyō-Roshi No. 51 Paper (40 × 40 cm). After application of the sample and drying, the sheet was developed for 15~16 hours at 23~24°C by ascending chromatography in a solvent system containing of *n*-butanol, acetic acid, and water (4 : 1 : 2). After development, the paper chromatogram was air-dried for 5~6 hours to remove acetic acid, and placed for 15 minutes on an agar plate (pH 6.4) which had been seeded with *S. lutea* PCI 1001. After the removal of the sheet, the plate was incubated at 37°C for 20 hours. Zones of inhibition of cephaloglycin and its metabolite, desacetylcephaloglycin, were clearly visible.

For a thin-layer chromatography, sample of cephaloglycin or desacetylcephaloglycin and the test samples of urine were applied to a thin-layer (Silica gel) chromatogram sheet. After the sheets were dried, the chromatogram was developed by ascending chromatography in solvent system consisting of ethyl acetate, acetic acetate, and water (3 : 1 : 1). After 3-hour development, the sheet was air-dried and placed on an agar plate (pH 6.4) which had been seeded with *S. lutea* PCI 1001. After 15 minutes the chromogram sheet was removed, and the plate was incubated at 37°C for 20 hours. Zones of inhibition of cephaloglycin or its metabolite were clearly visible.

Observations

Disagreement of Serum and Urine Cephaloglycin Determinations by *S. lutea* with those by *B. subtilis* or *S. hemolyticus*

Serum and urine samples from ten volunteers given single oral doses of cephalo-

Table 1. Bio-assay values in serum and urine using *Sarcina lutea*, *Streptococcus hemolyticus* and *Bacillus subtilis* as test organisms after a 500 mg oral administration of cephaloglycin to healthy volunteers

Test organisms	Serum (2 hrs) (mcg/ml)					Urine excretion (0~6 hrs) (mg)			
	A*	B*	C*	D*	E*	A*	B*	C*	D*
S. lutea PCI-1001	1.46	1.88	1.46	2.20	3.50	170.0	118.4	116.0	141.5
S. hemolyticus D	0.39	0.41	0.39	0.52	0.98	88.4	31.1	28.8	23.5
B. subtilis PCI-219	—	—	—	0.64	1.00	—	—	—	32.2
Ratios of sensitivities									
S. lutea/*S. hemolyticus*	3.7	4.6	3.7	4.2	3.6	1.9	3.7	4.0	6.0
S. lutea/*B. subtilis*	—	—	—	—	3.5	—	—	—	4.4
S. hemolyticus/*B. subtilis*	—	—	—	1.2	1.0	—	—	—	1.3

* Volunteers. —, not done.
Note: The values were calculated by dose-response curve of cephaloglycin used as standard. Methods of assay were carried out by cylinder cup-plate with *S. lutea* and *B. subtilis* and by superposition assay with *S. hemolyticus* as test organisms, respectively.

Table 2. Bio-assay values in serum and urine using *Sarcina lutea* and *Streptococcus hemolyticus* as test organisms after 500 mg oral administration of cephaloglycin to healthy volunteers

Case	Test organisms	Serum* (mcg/ml)				Urine** (mcg/ml)			
		1 hr.	2 hrs.	4 hrs.	6 hrs.	1 hr.	2 hrs.	4 hrs.	6 hrs.
Y	*S. lutea*	0.8	1.6	0.65	0.13	80	490	440	115
	S. hemolyticus	—	0.6	—	—	—	140	160	42
						(55 ml)	(105 ml)	(136 ml)	(120 ml)
A	*S. lutea*	—	1.25	0.31	—	—	440	1,650	130
	S. hemolyticus	—	—	—	—	—	180	700	30
						(40 ml)	(44 ml)	(48 ml)	(84 ml)
K	*S. lutea*	0.9	2.4	0.31	—	40	960	750	175
	S. hemolyticus	0.4	0.6	—	—	—	270	160	30
						(68 ml)	(60 ml)	(132 ml)	(68 ml)
S	*S. lutea*	0.48	1.6	1.9	0.48	40	290	760	660
	S. hemolyticus	—	1.2	1.2	—	38	160	380	430
						(120 ml)	(80 ml)	(130 ml)	(50 ml)
Sa	*S. lutea*	0.48	0.95	0.44	—	14	230	520	420
	S. hemolyticus	—	0.4	—	—	6	160	320	230
						(80 ml)	(60 ml)	(80 ml)	(40 ml)

* without dilution. ** diluted to 100-fold with buffer (pH 7.0).
( )=urine volume. —, not obtained.
Note: Same as Table 1.

glycin were assayed against a cephaloglycin standard and with the three test organisms (Tables 1 and 2). From the values listed in the tables, it will be noted that the values obtained with *B. subtilis* or *S. hemolyticus* were approximately $^1/_4$ of those obtained with *S. lutea*. These discrepancies suggest that the antibiotic undergoes modification *in vivo* to metabolites with different specific activities. Cephaloglycin, like cephalothin, possesses an acetyl group which can be removed by hydrolysis or by enzymatic esterase activity and it occurred to us that the observed discrepancies might be due to the desacetyl compound.

Bioautograph of a Paper and a Thin-layer Chromatogram of Cephaloglycin and Its Metabolite, Desacetylcephaloglycin, in Urine after Oral Administration of Cephaloglycin

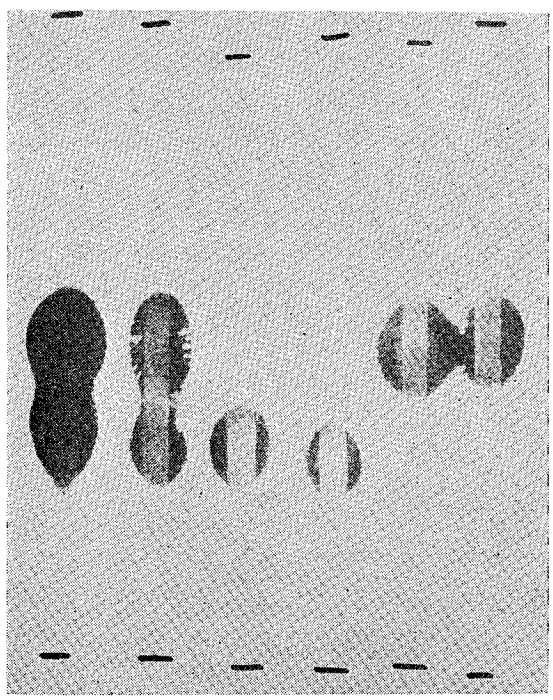
A bioautograph of a paper chromatogram demonstrating the separation of cephaloglycin and its metabolite is shown in Fig. 1. The bioautographic study was done at the time when we had not yet obtained desacetylcephaloglycin from Lilly Research Laboratory. As shown in this figure, the 0~2-hour sample produced two chromatographic zones, a smaller one with an Rf corresponding to that of cephaloglycin and a larger zone of a compound with greater mobility. The 0~1-hour sample revealed only the faster moving component. This can be interpreted to mean that almost all the cephaloglycin in urine, when given orally, is transformed into a different biological active metabolite with a different Rf from that of cephaloglycin.

Fig. 1. Bioautograph of metabolite of cephaloglycin after a 500 mg single oral administration into a healthy volunteer.

A =Cephaloglycin is dissolved in normal human urine (1.8 mcg/ml)
B_1=Human urine excretion 0~2 hours after oral administration of cephaloglycin
B_2=Human urine excretion 0~7 hours after oral administration of cephaloglycin

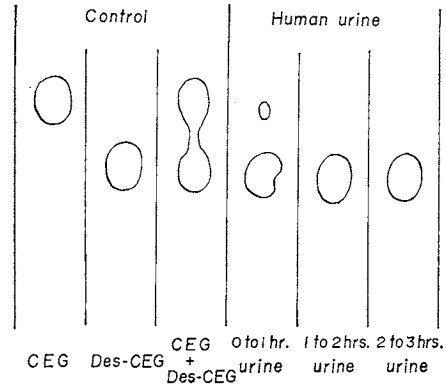
A B_1 B_2

Fig. 2. Bioautograph of standard preparation showing the separation of desacetylcephaloglycin (slower spot) from cephaloglycin.
Test organism : *B. subtilis* PCI-219
D=Desacetylcephaloglycin. C=Cephaloglycin.
( ) : indicates concentration (mcg/ml) of each antibiotic.



D+C (4+2) D+C (2+1) D (4) D (2) C (2) C (1)

Fig. 3. Bioautograph of a thin-layer chromatogram of cephaloglycin (CEG) and desacetylcephaloglycin (DES-CEG) after a 500 mg single oral administration into a healthy volunteer.



This Rf was later found to be identical to that obtained with desacetylcephaloglycin. A bioautograph of papergram of standard preparation demonstrating the separation of cephaloglycin and desacetylcephaloglycin is shown in Fig. 2.

A bioautograph obtained from a thin-layer chromatogram is shown in Fig. 3. The results after oral administration of cephaloglycin identical to those obtained in the paper chromatographic experiment in which almost all the antibiotic was transformed into desacetylcephaloglycin in urine.

Antibacterial Activity of Cephaloglycin and Desacetylcephaloglycin

The *in vitro* antibacterial activity of cephaloglycin and desacetylcephaloglycin was determined by the agar-streak dilution method. As can be seen from the date in Table 3, the activity of desacetylcephaloglycin against *B. subtilis*, *S. aureus* and *S. hemolyticus* is 0.1~0.5 that of cephaloglycin but against *S. lutea* the two compounds are of similar activity.

Table 3. Comparison of *in vitro* activity of cephaloglycin with desacetylcephaloglycin against various test organisms

Test organisms	MIC (mcg/ml of weight basis)	
	Desacetyl-cephaloglycin	Cephaloglycin
B. subtilis PCI-219	0.5	<0.05
Staph. aureus 209P	1.0	0.5
Sarc. lutea PCI 1001	<0.05	<0.05
St. hemolyticus D	0.5	0.1

Dose-response Curve of Cephaloglycin and Desacetylcephaloglycin against the Test Organisms in Cylinder-plate Diffusion

A dose-response curve was prepared to define the range over which the responses would be linear when plotted against logarithms of the concentrations of the antibiotics. The dose-response curves of cephaloglycin and desacetylcephaloglycin against *S. lutea*, *B. subtilis* and *S. hemolyticus* are shown in Figs. 4 and 5. Again, it is apparent that *S. lutea* PCI 1001 is highly sensitive to desacetylcephaloglycin. The inhibition zones with desacetylcephaloglycin were actually greater than those with cephaloglycin (Fig. 4). *B. subtilis* and *S. hemolyticus* are less sensitive to the desacetyl compound (Figs. 4 and 5).

Fig. 4. Dose-response curve of cephaloglycin (CEG) and desacetylcephaloglycin (DES-CEG) against *Sarcina lutea* and *Bacillus subtilis* by cylinder-plate assay method.

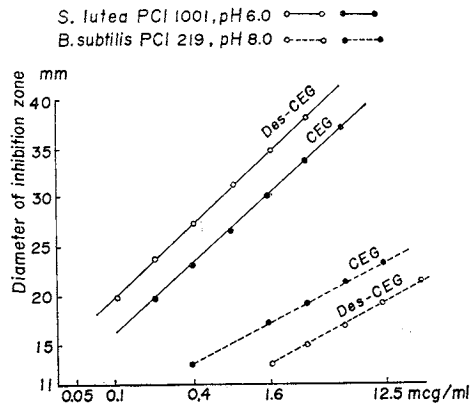
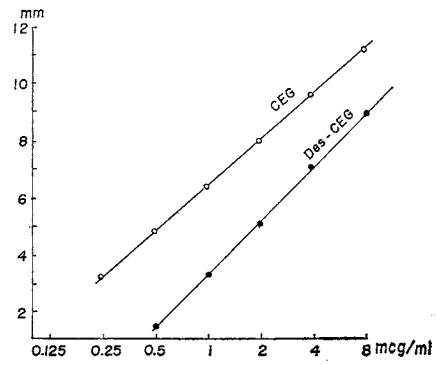


Fig. 5. Dose-response curve of cephaloglycin (CEG) and desacetylcephaloglycin (DES-CEG) against *Streptococcus hemolyticus* D by superposition assay method, using pH 6.4 medium.



This different specific activity for the both compounds is a most important factor in explaining the bio-assay discrepancy between test organisms. Lower values shown for *B. subtilis* or *S. hemolyticus* assay are due to parent compound, cephaloglycin, while greater values shown for *S. lutea* assay are due to the metabolite, desacetylcephaloglycin.

Assay of Cephaloglycin and Desacetylcephaloglycin Combinations by Cylinder-plate Diffusion Method

The cylinder-plate assay procedure with *S. lutea* and *B. subtilis* as test organisms was used to assay combinations of cephaloglycin and desacetylcephaloglycin. It was also apparent from the results shown in Table 4 that the cylinder-plate assay of cephaloglycin and desacetylcephaloglycin combinations could not measure the true total and each values of both antibiotics due to their different specific activities.

Table 4. Microbiological assay for cephaloglycin and desacetylcephaloglycin combinations by cylinder-plate diffusion method

Antibiotics in combination (mcg/ml)			Assay values (mcg/ml) from the following test organisms :			
			S. lutea		*B. subtilis*	
DES-CEG	CEG	Total	DES-CEG*	CEG**	DES-CEG*	CEG**
1.00	0.40	1.40	1.50	3.20	2.40	0.67
1.00	0.20	1.20	1.35	3.00	1.60	0.40
1.00	0.10	1.10	1.35	3.00	1.60	0.40
1.00	0.05	1.05	1.35	3.00	1.60	0.40
1.00	0	1.00	0.98	3.00	—	—

Note : DES-CEG=Desacetylcephaloglycin. CEG=Cephaloglycin.
* Concentration was calculated by DES-CEG standard curve.
** by CEG standard curve.
— not obtained.

Discussion

It is known that antibiotics may undergo metabolic changes in the body to give rise to metabolites which have antimicrobial activity by the formation of active matabolites of penicillins and desacetyl compound of cephalosporin antibiotic, cephalothin. Similarly, the new cephalosporin antibiotic, cephaloglycin, undergoes metabolic changes in the body to give rise to a desacetyl compound. From the present studies, we have found that almost all of the orally administered cephaloglycin when excreted in urine is transformed into desacetylcephaloglycin. The formation of the active metabolite is of significance from the point of view of assay of levels in body fluids because it is not valid to assay the levels simply using cylinder-plate diffusion procedure : the presence of the metabolite had an influence on the assay of the parent compound. From this reason, it is necessary to use paper chromatographic assay method involving a bioautographic technique. Recently, quantitative papergram and thin-layer assay methods for cephaloglycin and desacetyl compound were reported by HOEHN *et al.*[3]

Formation of the active metabolites is of significance from the point of view of chemotherapy because the desacetyl compound is less in activity than cephaloglycin.

Acknowledgements

We acknowledge the capable assistance of Dr. SABURO OKAMOTO, JUN ISONO and KIYOJI KANAZAWA, Shionogi Research Laboratory, in the performance of these studies.

References

1) WICK, W. E. & W. S. BONIECE : *In vitro* and *in vivo* laboratory evaluation of cephaloglycin and cephaloridine. Appl. Microbiol. 13 : 248~253, 1965
2) RONALD, A. & M. TURCK : Factors influencing *in vitro* susceptibility to the cephalosporins and clinical trial of an oral cephalosporin, cephaloglycin. Antimicr. Agents & Chemoth.-1966 : 82~87, 1967
3) HOEHN, M. M. & C. T. PUGH : Method for the detection and quantitative assay of cephaloglycin and its biologically active metabolites. Appl. Microbiol. 16 : 1132~1133, 1968