ACIDIC DEGRADATION OF CEPHALOGLYCIN AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DEACETYL-CEPHALOGLYCIN IN HUMAN URINE

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In order to provide fundamental knowledge about the determination of deacetyl-cephaloglycin excreted in human urine as an active metabolite of cephaloglycin, the degradation of cephaloglycin in acidic media has been investigated with varying reaction temperature between 30° and 50°C and pH between 1.2 and 2.8. The degradation pathway observed under these conditions was the elimination of the 3-acetyl group yielding deacetylcephaloglycin followed by formation of deacetylcephaloglycin lactone. Estimation of first order rate constants revealed that deacetylation is the rate-determining step for the degradation of cephaloglycin to the lactone. It is found from the kinetic results that reproducible assays of deacetylcephaloglycin excreted in urine can be achieved by a quantitative conversion to deacetylcephaloglycin lactone in a medium of pH 1.4 at 37°C for 2 hours, followed by a reversed-phase ion-pair high-performance liquid chromatography. The utility of the present method is demonstrated by determining the time course of urinary excretion of deacetyl-cephaloglycin after oral administration of cephaloglycin capsule.

Several investigations have reported the *in vitro* stability of cephalosporins[1~3]. It is known that in neutral and alkaline media cephalosporins as well as penicillins undergo facile cleavage of the β-lactam bond. 3-Acetoxymethylcephalosporins (*e.g.* cephaloglycin, cephalothin), unlike 3-deacetoxy derivatives (*e.g.* cephalexin, cephradine), have another possible site susceptible to degradation in acidic media. YAMANA and TSUJI[4] have shown that the acidic hydrolysis of 3-acetoxymethyl-cephalosporins yields corresponding deacetyl intermediates which further undergo rapid lactonization. Such reactivity has been utilized for the syntheses of 3-deacetyl derivative and corresponding lactone[5]. The acidic degradation kinetics, however, were quantified only by following the decrease in the amount of unchanged species[4], and no kinetic consideration has been made on the formation and disappearance of deacetylcephaloglycin and corresponding lactone.

The *in vivo* studies on the fate of cephaloglycin in man have revealed that a small portion of an orally administered amount is excreted in urine in the intact form[6], and deacetylcephaloglycin is the sole known metabolite[7]. The hydrolysis of side chain amide linkage to form 2-phenylglycine has also been known as another metabolic pathway in the rat dosed with cephaloglycin-^{14}C.[8] The analytical methods used in these studies, differential microbioassay and bioautography, involve a problem that deacetylcephaloglycin retains anti-microbe activity comparable to cephaloglycin itself[7,9], requiring consequent tedious procedure.

In our studies on chromatographic determination and pharmacokinetic analysis of β-lactam antibiotics[10~12], a simple and accurate method for the assay of cephaloglycin and its metabolites was needed. We intended at first to determine the amount of deacetylcephaloglycin in urine by referring to its standard material. Deacetylcephaloglycin, however, was hard to obtain in a pure form possibly due

to chemical instability. The published method of synthesis[5] afforded a very low yield of crude material which was hard to purify. Thus, we attempted to quantitate by converting deacetylcephaloglycin to corresponding stable lactone. This paper describes the acidic degradation of cephaloglycin and the determination of deacetylcephaloglycin by high-performance liquid chromatography.

Experimental

Reagent and Materials

Cephaloglycin used as a standard material (1,010 μg/mg as potency) and cephaloglycin capsule (Kefglycin® 250 mg as potency) received by volunteers were gifts from Shionogi Seiyaku Co. (Osaka, Japan). Deacetylcephaloglycin lactone was synthesized by the established method[5] and its purity was confirmed by elemental analysis and high-performance liquid chromatography. Sodium *n*-heptylsulfonate used as an ion-pairing agent was synthesized according to Strecker reaction[13]. Glass distilled water and methanol were degassed and used to prepare the mobile phase.

Chromatography

A high performance liquid chromatograph (Waters Assoc., ALC/GPC) equipped with a UV detector (254 nm) was used with a stationary phase of μ-Bondapak C_{18}®(Waters Assoc.) packed in a 4-mm × 30-cm stainless steel tubing and a mobile phase of water - methanol (4: 1, v/v) containing 0.004 M sodium *n*-heptylsulfonate and 0.01 M $(NH_4)H_2PO_4$, whose flow rate was maintained at 1.0 ml/min (1,400 psi.). A short column (4 mm × 2 cm) packed with Bondapak C_{18}/Corasil B (Waters Assoc.) was used to guard the main column.

Kinetic Measurement

The known amounts of standard cephaloglycin and deacetylcephaloglycin lactone were dissolved in normal human urine to make the concentrations 175.4 μg/ml and 445 μg/ml, respectively. With deacetylcephaloglycin, human urine collected from a volunteer (T. N.) 3 hours after oral administration of 1 g cephaloglycin was used as a substitute for standard solution. The concentration of deacetylcephaloglycin in this urine was later estimated to be 880 μg/ml. To a 1-ml portion of each urine solution was added an appropriate volume of 0.1 N HCl to adjust pH to 1.2, 1.4, 1.8, or 2.8. The acidified solution was sealed in a glass vessel and put in a thermostatic water-bath whose temperature was maintained at 30°, 37°, or 50°C. A small portion of the reaction solution was withdrawn at an appropriate time interval and submitted to high-performance liquid chromatography under the conditions described above. The rate constants were estimated as the slope of time vs. logarithmic residual or productive % plots for each species.

Urinary excretion

The cephaloglycin capsules (250 mg × 4) were orally administered to each of three male volunteers who had been fasted for 12 hours before receiving the capsules. Urine samples were collected just before and 1, 2, 3, 4, 6, 8, and 10 hours after administration. After measuring the volume, the urine was filtered through 0.45 μm pore size triacetylcellulose filter (Fuji Photo Film Co. Tokyo, Japan). A 1.0-ml portion of the filtrate was acidified to pH 1.4 by addition of a known volume of 0.1 N HCl, and kept at 37°C for 2 hours. A 2- ~10-μl portion of the reaction solution was exactly applied to the liquid chromatograph. The quantitations of deacetylcephaloglycin were achieved by referring to the calibration graph constructed on the basis of peak heights for the several known concentrations of standard deacetylcephaloglycin lactone dissolved in control urine. The empirical equation for the conversion was [deacetylcephaloglycin] = 1.027[deacetylcephaloglycin lactone], where [ ] denotes a concentration unit, weight/volume.

Results and Discussion

Prior to the kinetic measurements in urine solution, the degradation of cephaloglycin in acidic aqueous solution was investigated. On a chromatogram obtained from a pH 1.3 solution of cephalogly-

cin, there appeared only the peaks due to cephaloglycin and deacetylcephaloglycin lactone in the whole monitored duration of the reaction at 37°C. The relative intensity of these two peaks changed as a function of reaction time, whereas no peak assignable to deacetylcephaloglycin was observed within the full-scale recorder response for the peaks of cephaloglycin and deacetylcephaloglycin lactone. This is because, as mentioned later, deacetylcephaloglycin is lactonized at a much higher rate than is formed from cephaloglycin. The hydrolysis of side chain amide linkage to form 7-aminocephalosporanic acid was unlikely; otherwise it could have been detected by the particular UV wave length (254 nm). The cleavage of β-lactam bond was suspensive as far as chromatographic observation was concerned, since it would cause the UV absorbance to be markedly decreased. Stability investigations on cephalosporin C[1] and cephalothin[4], however, suggest that the cleavage of β-lactam bond in an acidic solution may be much slower than deacetylation.

The degradation of cephaloglycin in urine solution was also followed by high-performance liquid chromatography. The standard cephaloglycin dissolved in normal urine without addition of 0.1 N HCl gave a peak completely separated from the background peaks due to regular urinary components. The intensity of the peak was quite reproducible against the repeated injection of a constant volume, and no extra peaks appeared on the chromatogram when the solution was kept frozen. On acidifying the solution, an additional peak due to deacetylcephaloglycin lactone was eluted after the peak of cephaloglycin, whereas no peak due to deacetylcephaloglycin was again obtained and no change was observed in the background peaks. Fig. 1 shows the chromatogram of the intact urine excreted after administration of cephaloglycin, where in order to clear the separation profile the sensitivity was raised by 40-times after elution of deacetylcephaloglycin peak. The interesting facts found in Fig. 1 are that the peak assigned to deacetylcephaloglycin is eluted with a peak height over

Fig. 1. Separation of cephaloglycin (2), deacetylcephaloglycin (1) and deacetylcephaloglycin lactone (3) from regular urinary components.

Sample is the urine collected from a volunteer (T. N.) 3 hours after administration of 1 g cephaloglycin capsule; the concentrations of cephaloglycin, deacetylcephaloglycin and deacetylcephaloglycin lactone were estimated to be 8.34 μg/ml, 880 μg/ml and 7.24 μg/ml, respectively.

Injected volume; 5 μl.

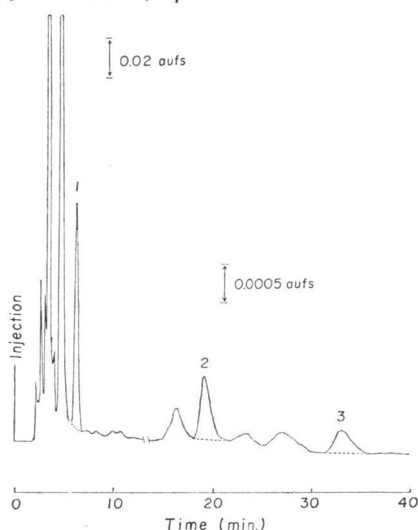
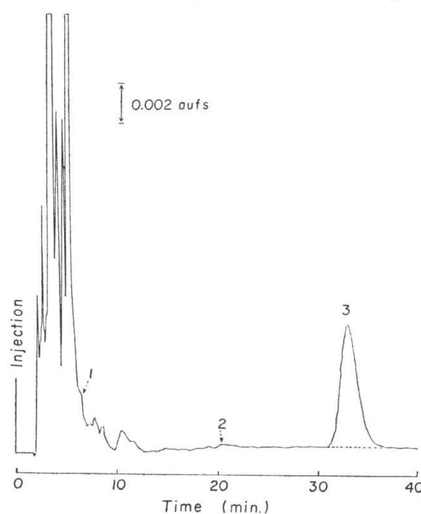


Fig. 2. Conversion of deacetylcephaloglycin to deacetylcephaloglycin lactone in acidified urine (pH 1.4, 37°C, 2 hours).

Injected volume; 5 μl. Sensitivity; 0.02 aufs.

Peak numbers correspond to those in Fig. 1.



200-times higher than that of unchanged cephaloglycin, and that the minor peak of deacetylcephaloglycin lactone is detected from the unacidified urine. When the urine sample was acidified, as seen in Fig. 2, the former peak disappeared more rapidly than the peak of cephaloglycin, accompanied with a noticeable increase in the peak intensity of deacetylcephaloglycin lactone. These results confirmed the peak assignment of deacetylcephaloglycin and that deacetylcephaloglycin lactone is a minor metabolite of cephaloglycin which is found for the first time from human urine. The peak intensity of deacetylcephaloglycin lactone obtained thereby would be partly due to the amount which had been originally present in urine as the metabolite and to that formed from unchanged cephaloglycin. These amounts, however, were negligibly small compared with that from deacetylcephaloglycin.

Scheme 1. Degradation pathway of cephaloglycin in acidified urine.

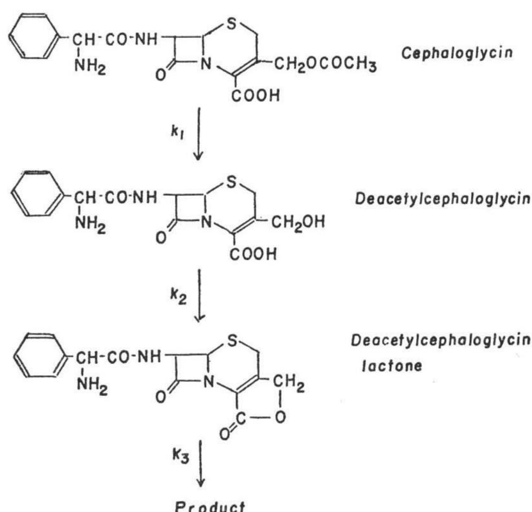
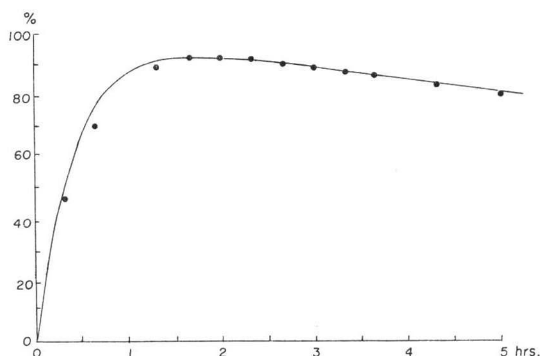


Fig. 3. Simulation curve for yield (%) of deacetylcephaloglycin lactone in acidified urine (pH 1.4, 37°C).

Closed circles indicate experimental data points.



Scheme 1 illustrates the degradation pathway of cephaloglycin thus confirmed in acidified urine, where k_1 and k_2 are the rate constants for deacetylation and lactonization, respectively. Although the cleavage of β-lactam bond may be involved concurrently with k_1 and k_2 steps, it is expected, as mentioned above, to give a smaller contribution than k_1 and k_2 to the overall kinetics. The time vs. lactonization % plots of deacetylcephaloglycin in acidified urine (Fig. 3) indicated a maximum followed by a gradual decrease. This downgrade was very close to the slope of the time course obtained from acidified control urine solution of standard deacetylcephaloglycin lactone. Therefore, k_3 was approximated to this slope. The peak of the degradation product, however, was not observed on the chromatogram. The rate constants, k_1 and k_2, estimated from the residual % plots for cephaloglycin and deacetylcephaloglycin, respectively, are listed in Table 1, where the values for k_2 at 50°C were not obtained, because the lactonization rate at this temperature was too fast to be followed by the present method.

In line with another purpose of this paper, that is, high performance liquid chromatographic determination of deacetylcephaloglycin in urine, the relevant kinetic step involving k_2 and k_3 was followed by simulation. When the initial mole concentration of deacetylcephaloglycin in urine, C_0, is decreased by the consecutive acid-catalyzed degradation as shown in Scheme 1, it follows that

$$[\mathrm{DACEGL}]/C_0 = k_2(k_3 - k_2)^{-1}(e^{-k_2 t} - e^{-k_3 t}),$$

where [DACEGL] designates the mole concentration of deacetylcephaloglycin lactone at time t after acidification of urine. Fig. 3 shows a simulation curve obtained by substituting the values for k_2 and k_3 in Table 1 (37°C, pH 1.4) to the above equation, indicating that the curve is well fitted to the experimental data points and the maximum yield of deacetylcephaloglycin lactone is estimated to be 93% theoretical at 1.7 hours. The reaction time varied between 100 and 140 minutes produced insignificant difference in the yield %, therefore being flexible for the *in vivo* assay. The reaction condition for the assay of deacetylcephaloglycin described in experimental part was thus determined as pH 1.4, 37°C and 2 hours, and the empirical factor (1.027) was calculated by substituting the k_2, k_3, and t values at this condition to the right hand side of the above equation. The overall reproducibility of the present method was found to give coefficient of variation 0.7% for 880 μg/ml (n=4).

The assay method thus established was applied to the *in vivo* experiments. Figs. 4 and 5 show the time courses of urinary excretion rate and cumulative excretion amount of deacetylcephaloglycin for

Table 1. Rate constants for the degradation of cephaloglycin, deacetylcephaloglycin, and deacetylcephaloglycin lactone.

Temperature	pH	hrs.$^{-1}$		
		k_1	k_2	k_3
30°C	1.2	0.019	1.538	0.018
	1.4	0.020	1.197	0.013
	1.8	0.009	0.496	0.016
	2.8	0.006	0.080	0.014
37°C	1.2	0.054	3.885	0.040
	1.4	0.053	2.439	0.043
	1.8	0.038	1.021	0.032
	2.8	0.034	0.176	0.024
50°C	1.2	0.162	—	0.120
	1.4	0.161	—	0.112
	1.8	0.115	—	0.102
	2.8	0.084	—	0.121

Fig. 4. Time course of urinary excretion rate of deacetylcephaloglycin after oral administration of 1 g cephaloglycin capsule.

T.N., J.H. & M.M. specify the volunteers.

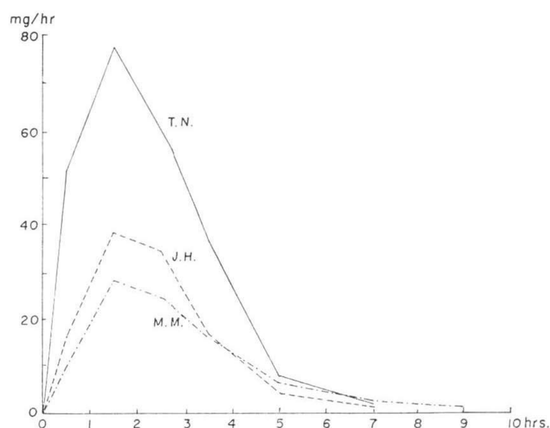
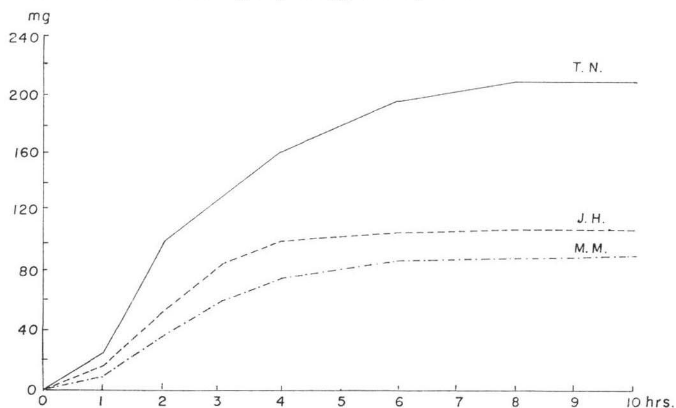


Fig. 5. Time course of cumulative urinary excretion amount of deacetylcephaloglycin after oral administration of 1 g cephaloglycin capsule.



three volunteers dosed with 1 g cephaloglycin capsule, respectively. The results reveal an appreciable scatterness due to individual difference among volunteers. The excretion ratio of deacetylcephaloglycin to the dosed amount at 10 hours after administration where the excretion is almost completed is 10.7 to 24.0% by cephaloglycin equivalent. The time course data to be compared with the present results have not appeared in literature except a paper given by SHIMIZU *et al.*[9], which indicates that average 26% of the administered amount is excreted in urine over 6 hours collection period. This value, however, is not due to deacetylcephaloglycin alone but to total activity against test organism (*Sarcina lutea*) which can detect both cephaloglycin and deacetylcephaloglycin[7]. The detailed discussion on the entire metabolic pathway and pharmacokinetic treatments of cephaloglycin will be given elsewhere.

References

1) KONECNEY, J.; E. FELBER & J. GRUNNER: Kinetics of the hydrolysis of cephalosporin C. J. Antibiotics 26: 135~141, 1973
2) INDELICATO, J. M.; T. T. NORVILAS, R. R. PHEIFFER, W. J. WHEELER & W. L. WILHAM: Substituent effects upon the base hydrolysis of penicillins and cephalosporins. Competitive intramolecular nucleophilic amino attack in cephalosporins. J. Med. Chem. 17: 523~527, 1974
3) YAMANA, T.; A. TSUJI, K. KANAYAMA & O. NAKANO: Comparative stability of cephalosporins in aqueous solution. J. Antibiotics 27: 1000~1001, 1974
4) YAMANA, T. & A. TSUJI: Comparative stability of cephalosporins in aqueous solution: Kinetics and mechanisms of degradation. J. Pharm. Sci. 65: 1563~1574, 1976
5) KUKOLJA, S.: Chemistry of cephalosporin antibiotics. XI. Preparation and properties of deacetyl-cephaloglycin and its lactone. J. Med. Chem. 11: 1067~1068, 1968
6) GRIFFITH, R. S. & H. R. BLACK: Blood, urine and tissue concentrations of cephalosporin antibiotics in normal subjects. Postgrad. Med. J. February Suppl.: 32~40, 1971
7) WICK, W. E.; W. E. WRIGHT & H. V. KUDER: Cephaloglycin and its biological active metabolite desacetylcephaloglycin. Appl. Microbiol. 21: 426~434, 1971
8) 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
9) SHIMIZU, K. & H. NISHIMURA: Problems in the bio-assay of orally administered cephaloglycin in biological fluid and method for the detection of its metabolite, desacetylcephaloglycin. J. Antibiotics 23: 216~222, 1970
10) NAKAGAWA, T.; J. HAGINAKA, K. YAMAOKA & T. UNO: Direct high speed liquid chromatographic determination of cephalexin in urine. J. Chromatogr. 147: 509~512, 1978
11) NAKAGAWA, T.; J. HAGINAKA, M. MASADA & T. UNO: Gas chromatographic and gas chromatographic-mass spectrometric analysis of cephalexin. J. Chromatogr. 154: 264~266, 1978
12) NAKAGAWA, T.; J. HAGINAKA, K. YAMAOKA & T. UNO: High speed liquid chromatographic determination of cephalexin in human plasma and urine. J. Antibiotics 31: 769~775, 1978
13) REED, R. M. & H. V. TARTER: The preparation of sodium alkyl sulfonates. J. Am. Chem. Soc. 57: 570~571, 1935