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## Alkaline Degradation of Clavulanic Acid and High Performance Liquid Chromatographic Determination by Post-Column Alkaline Degradation

JUN HAGINAKA,\*<sup>a</sup> HIROYUKI YASUDA,<sup>a</sup> TOYOZO UNO,<sup>a</sup>  
and TERUMICHI NAKAGAWA<sup>b</sup>

Faculty of Pharmaceutical Sciences, Mukogawa Women's University,<sup>a</sup> 4-16 Edagawa-cho,  
Nishinomiya 663, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,<sup>b</sup>  
Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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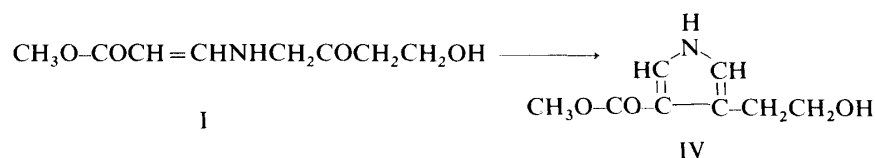
Alkaline degradation of clavulanic acid in methanol and in aqueous solution has been investigated. Potassium clavulanate was degraded in methanol and in NaOH-saturated methanol to yield methyl 8-hydroxy-6-oxo-4-aza-2-octenoate (I), which showed ultraviolet (UV) absorption with  $\lambda_{\max}$  268 nm (methanol). The UV absorption of I almost disappeared in acidic conditions, and reappeared on subsequent realkalization, suggesting interconversion between I and its protonated form. It was suggested that potassium clavulanate might be rapidly hydrolyzed in alkaline aqueous solutions to 8-hydroxy-6-oxo-4-aza-2-octenoic acid (II), which has strong UV absorption around 260 nm. The acid-base interconversion was also observed between II and its protonated form, the latter exhibiting almost no UV absorption around 260 nm.

An ion-pair reversed-phase high performance liquid chromatography method with the alkaline degradation reaction incorporated into post-column has been developed for the determination of clavulanic acid in plasma and urine. After separation from regular components of plasma and urine, clavulanic acid is degraded in NaOH solution in a reaction coil followed by detection of the UV absorbance of the degradation product at 270 nm. The procedure was quantitative over a wide range of clavulanic acid concentrations in plasma and urine down to 0.1  $\mu\text{g/ml}$ .

**Keywords**—clavulanic acid; clavulanic acid alkaline degradation; clavulanic acid degradation product UV spectra; clavulanic acid HPLC; post-column reaction

Clavulanic acid, *Z*-(2*R*, 5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid, is a potent inhibitor of various  $\beta$ -lactamases from clinical isolates.<sup>1)</sup> Therefore, the combined use of clavulanic acid with a certain class of penicillins and cephalosporins causes marked reduction in their minimum inhibitory concentrations *in vitro*<sup>2,3)</sup> and *in vivo*<sup>4-8)</sup> against various  $\beta$ -lactamase-producing bacteria.

In contrast with the extensive investigations on microbiological activity, some work has been done on the chemical properties of clavulanic acid. Stirling and Elson<sup>9)</sup> reported that the *p*-bromobenzyl ester of clavulanic acid is converted to a bicyclic lactone by ozonolysis, and to the corresponding *trans*-aminoacrylate by reaction with dibenzylamine in methanol. Bird *et al.*<sup>10)</sup> isolated the reaction product of clavulanic acid with imidazole, and identified it as 1-(8-hydroxy-6-oxo-4-aza-2-octenoyl)imidazole (III). Davies and Howarth<sup>11)</sup> reported that methanolysis of sodium clavulanate produces methyl 8-hydroxy-6-oxo-4-aza-2-octenoate (I), which undergoes base-catalyzed intramolecular condensation to afford methyl 4-(2-hydroxyethyl)-



pyrrole-3-carboxylate (IV). In the previous paper,<sup>12)</sup> we described the degradation of clavulanic acid in aqueous solutions over a pH range of 3.15 to 10.10 at ionic strength 0.5 at 35 °C. The pH vs. rate profiles indicated that the degradation in alkaline solutions proceeds, overall, about 10 times faster than in acidic media, the maximal stability being at pH 6.39.

The assays of clavulanic acid so far achieved in previous investigations have mainly been based on microbiological methods. Chemical and spectrometric methods were not available, possibly because clavulanic acid itself has no appreciable absorption in the ultraviolet (UV) region above 210 nm,<sup>13)</sup> and neither iodometric nor hydroxamate assays are suitable for quantitative purposes.<sup>14)</sup> In the previous paper,<sup>15)</sup> we described an ion-pair reversed-phase high performance liquid chromatography (HPLC) method with detection of UV absorbance enhanced by a bathochromic shift of  $\lambda_{\max}$  due to solvent effects. This method, even though the detection limit is as high as 5  $\mu\text{g}/\text{ml}$ , was used for investigations of stability in aqueous solutions<sup>12)</sup> and excretion in human urine following an oral dose of clavulanic acid alone or in combination with amoxicillin.<sup>16)</sup> However, for detailed pharmacokinetic investigations of clavulanic acid in man, it is necessary to develop a more sensitive and selective assay method. Thus, we attempted, first, to enhance the detectability of clavulanic acid by reaction of the 2-carboxylic acid group with *N,N*-dimethyl-4-diazomethyl benzenesulfonamide,  $\alpha$ ,*p*-dibromoacetophenone, 4-bromomethyl-7-methoxycoumarin, 1-bromoacetylpyrene, or 9-diazomethylanthracene, and by reaction of the hydroxyl group with phenylisocyanate or 3,5-dinitrobenzoyl chloride. These approaches were successful for other  $\beta$ -lactam antibiotics, but were found to be unsuitable for clavulanic acid. Then we tried to transform clavulanic acid to a UV absorbing or fluorescent product(s) by degradation under various conditions. An unknown fluorescent product was obtained when clavulanic acid was treated in alkaline solution (pH 12) at 60 °C.<sup>17)</sup> However, the fluorescent intensity was too weak to determine clavulanic acid at levels below 20  $\mu\text{g}/\text{ml}$ .<sup>17)</sup> Recently, a spectrometric method was developed by Bird *et al.*<sup>10)</sup> based on the absorption at 312 nm of the reaction product of clavulanic acid with imidazole. The method was applied to HPLC determination of clavulanic acid in human urine and plasma by Foulstone and Reading.<sup>18)</sup> Their HPLC procedure involved the ultrafiltration of serum samples and pre-column derivatization of clavulanic acid with imidazole.

The present paper deals with the alkaline degradation reaction of clavulanic acid in methanol and aqueous solution, and with post-column incorporation of the reaction in an HPLC method for the determination of clavulanic acid in plasma and urine at a level as low as 0.1  $\mu\text{g}/\text{ml}$  with minimum pretreatment.

### Experimental

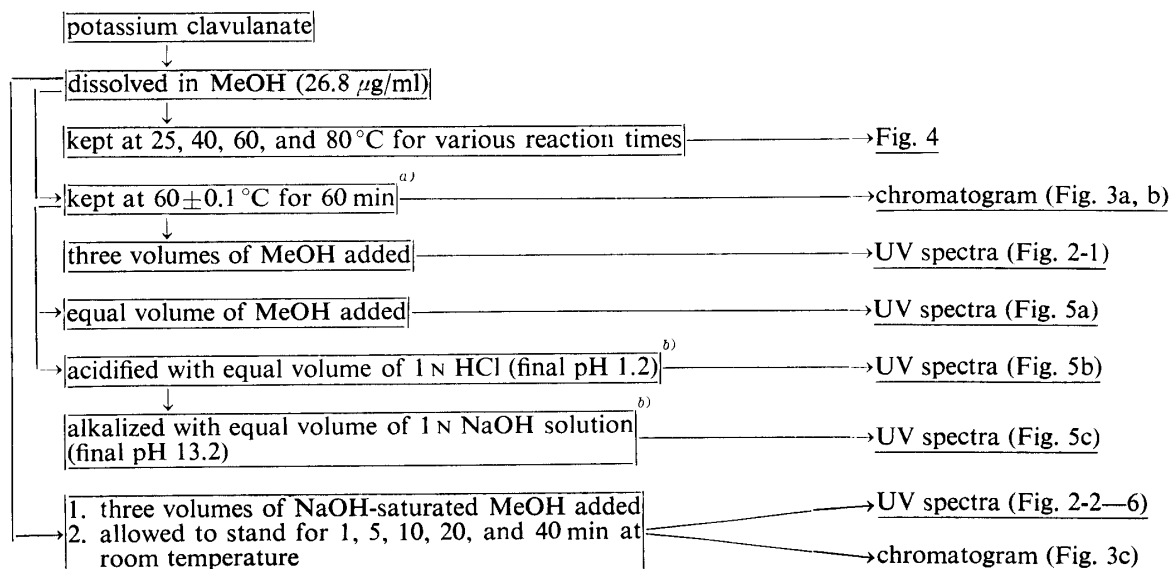
**Reagents and Materials**—Potassium clavulanate was supplied by Beecham Yakuhin Co., Ltd. (Tokyo, Japan). Tetrabutylammonium bromide (TBAB), buffer salts, and other chemicals of reagent grade were obtained from Nakarari Chemicals Co. (Kyoto, Japan). Deionized distilled water and distilled methanol were used for the preparations of mobile phase for HPLC and the solutions for measurements of UV spectra.

**pH Measurements**—The pH values were measured on a pH meter (HM-20E, Toa Electronics Ltd., Tokyo, Japan).

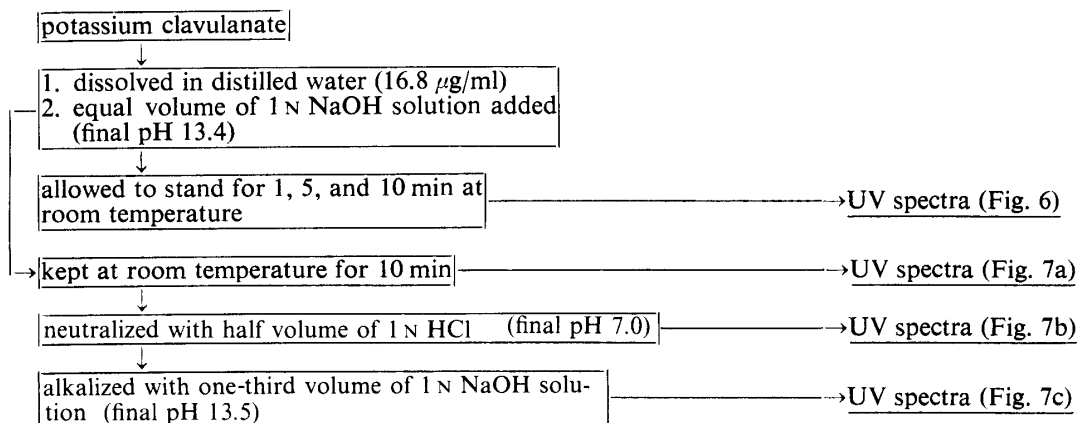
**Degradation of Clavulanic Acid**—The alkaline degradation of clavulanic acid was carried out according to the procedure depicted in Chart 1, which involves methanolysis, hydrolysis, reaction with imidazole followed by hydrolysis, and post-column reaction for HPLC.

**Isolation of the Degradation Product of Clavulanic Acid in Methanol**—Methyl 8-hydroxy-6-oxo-4-aza-2-octenoate (I): Potassium clavulanate (50 mg) was dissolved in 10 ml of methanol and kept in a glass vessel with a tight screw cap at  $60 \pm 0.1$  °C for 60 min. The reaction solution was cooled to room temperature and concentrated to about one-fifth of the initial volume under reduced pressure. The concentrated solution was subjected in portions to the chromatography under the following conditions in order to isolate the degradation product: stationary phase, Develosil ODS-10 (Nomura Chemicals Co., Seto, Japan) packed in 25 cm  $\times$  10 mm i.d. stainless steel tubing; mobile

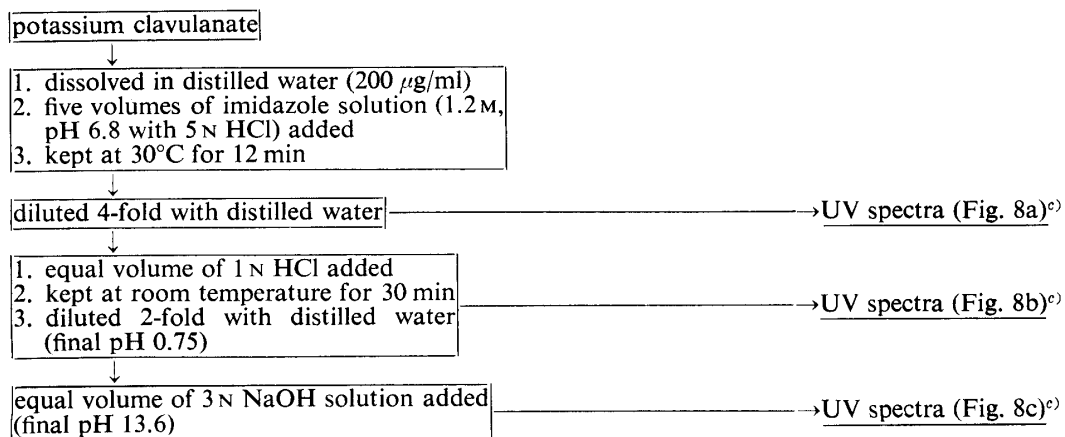
## (1) Methanolysis



## (2) Hydrolysis



## (3) Reaction with imidazole followed by hydrolysis



## (4) Post-column reaction for HPLC

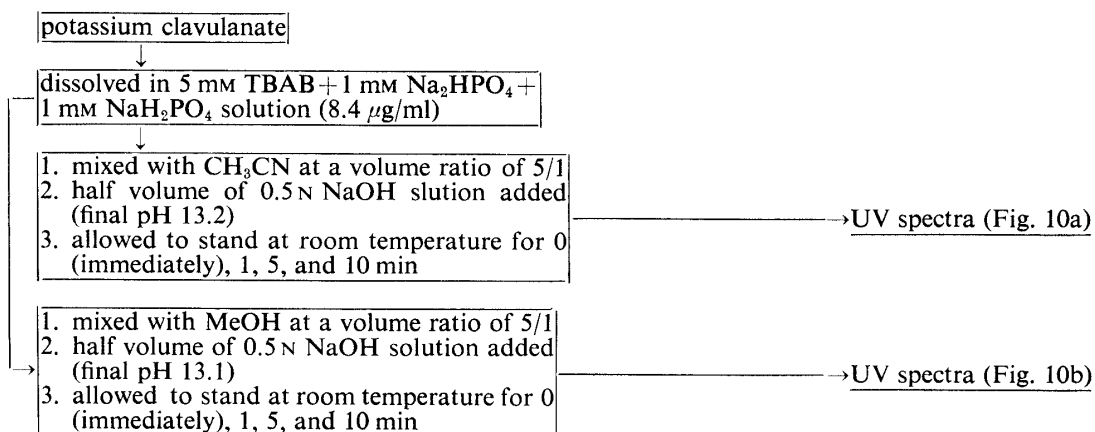


Chart 1. Procedures for Degradation of Clavulanic Acid

- a) Kept in a glass vessel with a tight screw cap.
- b) UV spectra were measured immediately.
- c) The intensities of UV spectra are comparable in every case.

phase, water/methanol=8/1 (v/v); flow rate, 3.0 ml/min; detection, UV 230 nm (UVIDEC-100-V, Jasco, Tokyo, Japan). The fraction with elution times between 30 and 40 min was collected. Evaporation of the solvent under reduced pressure at room temperature followed by lyophilization gave a yellowish-white solid. Field desorption mass spectrum (FD-MS):  $m/z$  187. Electron impact-mass spectrum (EI-MS):  $m/z$  187 (Obsd 187.0841, Calcd 187.0843).  $^1\text{H-NMR}$  ( $\text{CD}_3\text{COCD}_3$ )  $\delta$ : 2.72 (2H, t,  $J=6.2$  Hz,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 3.54 (3H, s,  $\text{CH}_3$ ), ca. 3.7 (1H, br, OH) (disappears on addition of  $\text{D}_2\text{O}$ ), 3.86 (2H, t,  $J=6.2$  Hz,  $\text{CH}_2\text{OH}$ ), 4.06 (2H, d,  $J=5.2$  Hz,  $\text{NHCH}_2$ ), 4.62 (1H, d,  $J=13.4$  Hz,  $\text{COCH=}$ ), 6.37 (1H, br, NH) (disappears on addition of  $\text{D}_2\text{O}$ ), 7.59 (1H, dd,  $J=13.4$  and 7.6 Hz,  $=\text{CHNH}$ ).  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{COCD}_3$ )  $\delta$ : 43.4 (t,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 50.7 (q,  $\text{CH}_3$ ), 53.9 (t,  $\text{NHCH}_2$ ), 57.6 (t,  $\text{CH}_2\text{OH}$ ), 85.5 (d,  $\text{COCH=}$ ), 150.6 (d,  $=\text{CHNH}$ ), 171.1 (s,  $\text{COCH=}$ ), 206.5 (s,  $\text{CH}_2\text{CO}$ ).  $\lambda_{\text{max}}$  (MeOH): 268 nm.

Methyl 4-(2-Hydroxyethyl)pyrrole-3-carboxylate (IV): Potassium clavulanate (125 mg) was dissolved in 100 ml of NaOH-saturated methanol and kept at room temperature for 30 min. The solvent was removed by evaporation under reduced pressure, and the residue was extracted with ethyl acetate. After removal of the solvent by evaporation, the residue was dissolved in a small volume of methanol, and subjected to HPLC separation under the same conditions as described above, except that the mobile phase of water/methanol=4/1 (v/v) was used. The fraction with elution times between 20 and 30 min was collected. Evaporation of the solvent under reduced pressure followed by lyophilization gave a reddish-yellow solid. FD-MS:  $m/z$  169. EI-MS:  $m/z$  169 (Obsd 169.0736, Calcd 169.0737).  $^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ )  $\delta$ : 2.92 (2H, t,  $J=7.3$  Hz,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 3.70 (2H, t,  $J=7.3$  Hz,  $\text{CH}_2\text{OH}$ ), 3.75 (3H, s,  $\text{CH}_3$ ), 6.61 (1H, d,  $J=2.2$  Hz,  $\text{CH=C-CH}_2\text{CH}_2\text{OH}$ ), 7.34 (1H, d,  $J=2.2$  Hz,  $\text{CH=C-COOCH}_3$ ).  $^{13}\text{C-NMR}$  ( $\text{CD}_3\text{OD}$ )  $\delta$ : 30.6 (t,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 51.1 (q,  $\text{CH}_3$ ), 64.0 (t,  $\text{CH}_2\text{OH}$ ), 114.3 (s,  $\text{C-COOCH}_3$ ), 119.3 (d,  $\text{CH=C-CH}_2\text{CH}_2\text{OH}$ ), 122.7 (s,  $\text{C-CH}_2\text{CH}_2\text{OH}$ ), 126.2 (d,  $\text{CH=C-COOCH}_3$ ), 168.0 (s,  $\text{C=O}$ ).  $\lambda_{\text{max}}$  (MeOH): 231 nm.

**Measurements of UV Spectra**—UV spectra were measured on a model UV-240 spectrophotometer (Shimadzu Co., Kyoto, Japan) using reference solution without potassium clavulanate.

**Chromatography**—Figure 1 depicts a schematic diagram of the instrumentation used for HPLC separation and post-column degradation of clavulanic acid. Pump A (TRIROTAR III, Jasco) was used for delivering the mobile phase, and pump B (TWINCLE, Jasco) for delivering aqueous NaOH solution to the reaction coil (2 m  $\times$  0.25 mm i.d.). The stationary phase used was Develosil ODS-10 (Nomura Chemicals Co.) packed in 25 cm  $\times$  4.6 mm i.d. stainless steel tubing. A short pre-column (5 cm  $\times$  4.6 mm i.d.) packed with LiChrosorb RP-2 (E. Merck, Darmstadt, West Germany) was used to guard the main column. The mobile phase used was 5 mM TBAB+0.1 mM  $\text{Na}_2\text{HPO}_4$ +0.1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH=3/1 (v/v) for plasma samples, and 5 mM TBAB+1 mM  $\text{Na}_2\text{HPO}_4$ +1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH=5/1 (v/v) for urine samples. The flow rate of the mobile phase was maintained at 1.2 ml/min. A 0.5 N NaOH solution was used for the post-column reaction at a flow rate of 0.6 ml/min. The detection wavelength was 270 nm. All chromatographic operations were performed at ambient temperature.

**Preparations of Urine and Plasma Samples**—A urine sample was passed through a 0.45  $\mu\text{m}$  pore size membrane filter (Fuji Photo Film Co., Ltd., Tokyo, Japan) and an appropriate portion of the filtrate was introduced accurately into the chromatograph under the conditions described above. A plasma sample mixed with two volumes of  $\text{CH}_3\text{CN}$  was shaken vigorously in a Vortex mixer for 30 s followed by centrifugation at 3600 rpm for 5 min. An appropriate volume of supernatant was accurately introduced into the chromatograph.

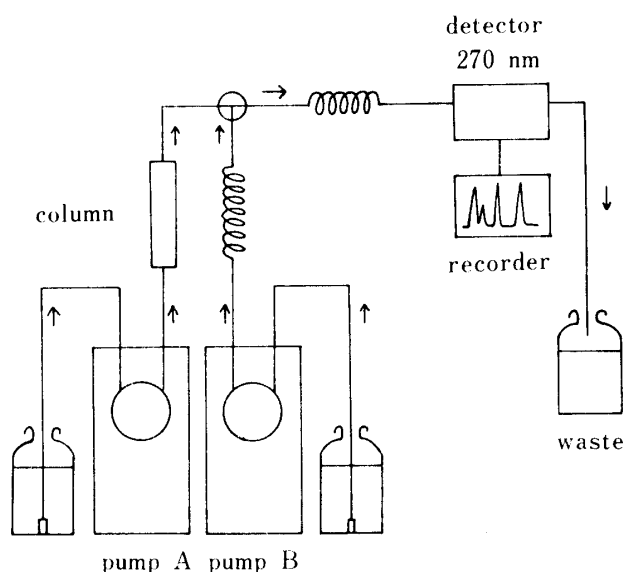


Fig. 1. Schematic Diagram of Instrumentation Used for HPLC Separation of Clavulanic Acid

Pump A, pump for delivering mobile phase; pump B, pump for delivering NaOH solution; column, Develosil ODS-10 (25 cm  $\times$  4.6 mm i.d.); detection, UV 270 nm.

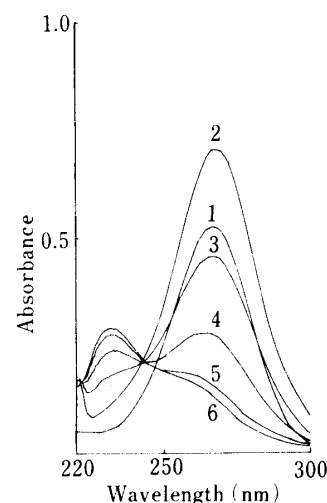


Fig. 2. UV Absorption Spectra of Clavulanic Acid after Degradation in Methanol and in NaOH-Saturated Methanol.

Reaction time: 1, 60 min; 2, 1 min; 3, 5 min; 4, 10 min; 5, 20 min; 6, 40 min. Sample preparations; see Chart 1.

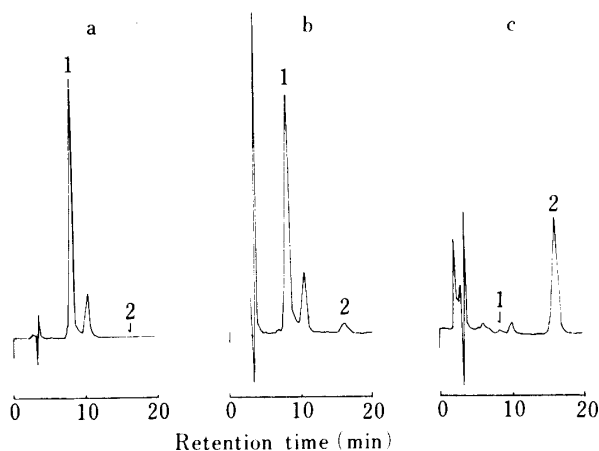


Fig. 3. Chromatogram of Clavulanic Acid after Degradation in Methanol and in NaOH-Saturated Methanol

Sample preparations: clavulanic acid (26.2  $\mu$ g/ml) was degraded in methanol at 60  $^{\circ}$ C for 60 min (a, b), and degraded in NaOH-saturated methanol for 40 min (c). For detailed procedure, see Chart 1. HPLC conditions: stationary phase, Develosil ODS-10 (25 cm  $\times$  4.6 mm i.d.); mobile phase, 2.5 mM  $\text{Na}_2\text{HPO}_4$  + 2.5 mM  $\text{NaH}_2\text{PO}_4$ /MeOH = 3/1 (v/v) (pH 7.34); flow rate, 1.0 ml/min; detection, 270 nm (a), 230 nm (b, c); sensitivity, 0.032 au (a), 0.008 au (b, c); injection volume, 5  $\mu$ l (a, c), 20  $\mu$ l (b). Peak 1: methyl 8-hydroxy-6-oxo-4-aza-2-octenoate (I). Peak 2: methyl 4-(2-hydroxyethyl)pyrrole-3-carboxylate (IV). A minor peak between peaks 1 and 2; unknown. Assignments, see the text.

## Results and Discussion

### Degradation of Clavulanic Acid in Methanol

Figure 2 shows the UV spectra of potassium clavulanate kept in methanol at 60  $^{\circ}$ C for 60 min and kept in NaOH-saturated methanol for 1, 5, 10, 20, and 40 min at room temperature. The spectra indicate that potassium clavulanate, which has no UV absorption above 210 nm,<sup>13</sup> is degraded in pure methanol to yield product(s) with  $\lambda_{\text{max}}$  268 nm. It was also found that there are at least two different UV absorbing products with  $\lambda_{\text{max}}$  268 and 230 nm in NaOH-saturated methanol and the intensity at 268 nm decreases with concomitant increase of absorbance at 230 nm as the reaction proceeds. The HPLC analysis of the methanolic solution shows two distinct peaks on the chromatogram (Fig. 3), where peak 1 exhibits a higher response at 270 nm than at 230 nm, while peak 2 is not detected at 270 nm. Peak 1 was obtained when potassium clavulanate was degraded in methanol (Fig. 3a, b), but disappeared on degradation in NaOH-saturated methanol (Fig. 3c). The retention times of

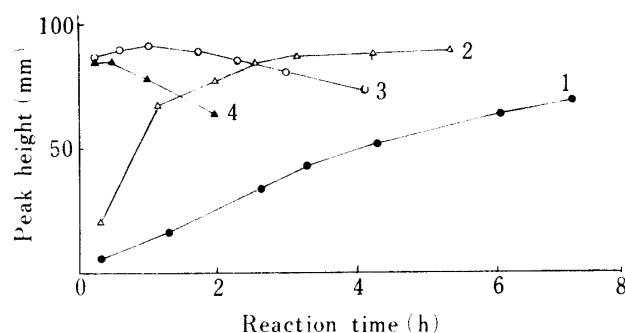


Fig. 4. Dependence of Methanolysis of Clavulanic Acid on Temperature

Clavulanic acid ( $26.2 \mu\text{g/ml}$ ) was degraded in methanol at various temperatures (1,  $25^\circ\text{C}$ ; 2,  $40^\circ\text{C}$ ; 3,  $60^\circ\text{C}$ ; 4,  $80^\circ\text{C}$ ). An appropriate volume ( $5\text{--}20 \mu\text{l}$ ) of the reaction solution was introduced into the chromatograph under the same conditions as described in Fig. 3. Peak height (peak 1) was plotted against reaction time.

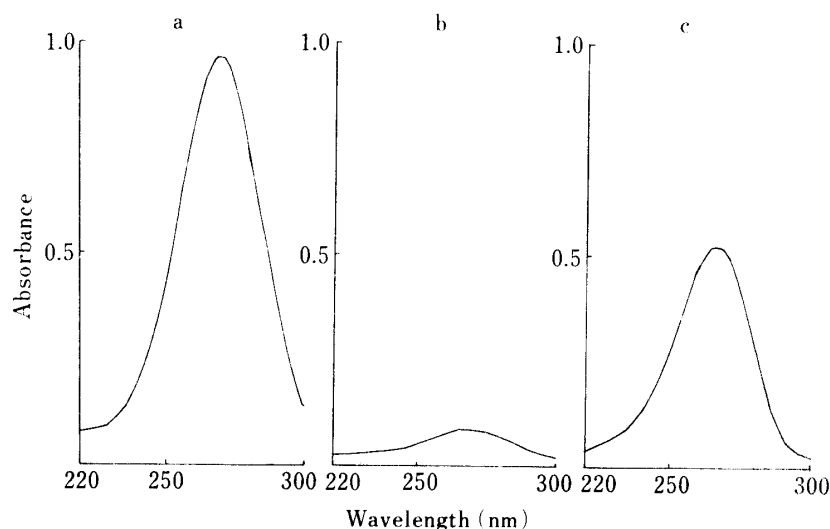


Fig. 5. pH-Dependent Spectral Change of Clavulanic Acid after Degradation in Methanol

Clavulanic acid ( $26.2 \mu\text{g/ml}$ ) was degraded in methanol at  $60^\circ\text{C}$  for 60 min. A portion of the reaction solution was diluted with an equal volume of methanol (a). Another portion of the reaction solution was acidified with an equal volume of  $1 \text{ N HCl}$  (final pH 1.2) (b), and the acidic solution was alkalinized with an equal volume of  $1 \text{ N NaOH}$  solution (final pH 13.2) (c).

the isolated substances I and IV agreed with those of peaks 1 and 2, respectively, in the mobile phase system described in Fig. 3. Therefore, the substances in peaks 1 and 2 in Fig. 3 are assigned as I and IV, respectively. The rate profile of the formation of I in pure methanol is shown in Fig. 4, where the reaction temperature was varied from  $25$  to  $80^\circ\text{C}$ . The reaction rate was markedly accelerated at elevated temperature. The maximum yield was attained at about 3 h after initiation of the reaction when the reaction temperature was  $40^\circ\text{C}$ , while it took 1 h or less to obtain almost the same magnitude of yield when the reaction temperature was raised to  $60\text{--}80^\circ\text{C}$ . However, a gradual decline of the yield following the maximum was observed at  $60$  and  $80^\circ\text{C}$ , suggesting intramolecular condensation to IV. This intramolecular condensation reaction is accelerated by the presence of  $\text{NaOH}$ , as reported by Davies and Howarth,<sup>11)</sup> and also by elevated temperature. It is noteworthy here that potassium clavulanate remained stable for several hours in 50% aqueous methanol at room temperature. Figure 5 shows the pH-dependent spectral change of potassium clavulanate after degradation

in methanol at 60 °C for 60 min. When the solution was acidified with HCl to pH 1.2 (Fig. 5b), the UV absorption at 268 nm (Fig. 5a) almost disappeared. Subsequent realkalization of the acidic solution to pH 13.2 by addition of NaOH solution resulted in reappearance of the UV absorption (Fig. 5c). It is known that  $\beta$ -aminoalkyl- $\alpha,\beta$ -unsaturated ketones show UV absorption at much longer wavelength than  $\beta$ -alkyl- $\alpha,\beta$ -unsaturated ketones because of strong resonance with the carbonyl.<sup>19)</sup> Therefore, it is conceivable that I has UV absorption at 268 nm, but the protonated form of I has almost no UV absorption at 268 nm.

#### Alkaline Degradation of Clavulanic Acid in Aqueous Solutions

Figure 6 shows the changes of UV spectra of potassium clavulanate after degradation in aqueous solutions (pH 13.4) at ambient temperature. Potassium clavulanate was rapidly degraded to yield product(s) having a UV absorption maximum around 260 nm. The degradation product(s) were fairly stable in the alkaline solution. Alkaline degradation for 10 min at pH 13.4 at room temperature (Fig. 7a) followed by neutralization with HCl to pH 7.0 resulted in the disappearance of UV absorption (Fig. 7b). When the neutral solution of

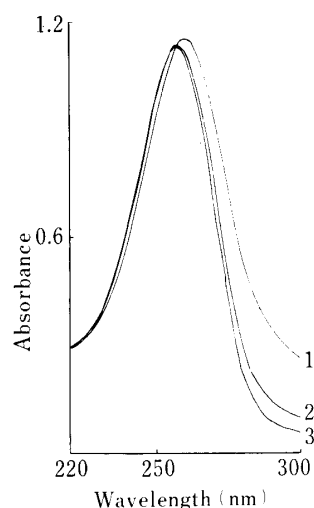


Fig. 6. UV Absorption Spectra of Clavulanic Acid after Degradation in Alkaline Aqueous Solutions at Various Reaction Times

Initial concentration of clavulanic acid, 16.8  $\mu\text{g/ml}$ ; pH of the reaction solution, 13.4. Reaction time: 1, 1 min; 2, 5 min; 3, 10 min.

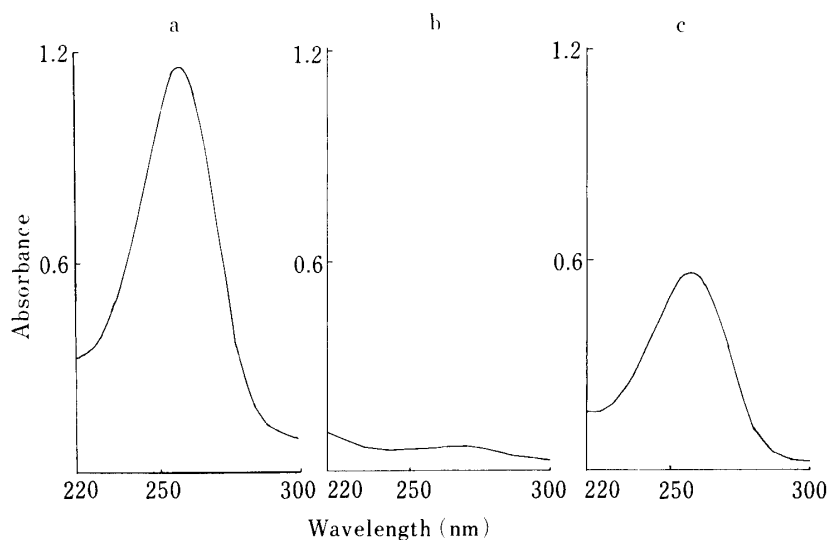


Fig. 7. UV Absorption Spectra of Alkaline Degradation Product of Clavulanic Acid in Alkaline, Neutral, and Realkalized Aqueous Solutions

Clavulanic acid (16.8  $\mu\text{g/ml}$ ) was reacted with an equal volume of 1 N NaOH solution for 10 min at room temperature (a). The alkaline solution was neutralized with a half volume of 1 N HCl (b). The neutral solution was realkalized with a one-third volume of 1 N NaOH solution (c).

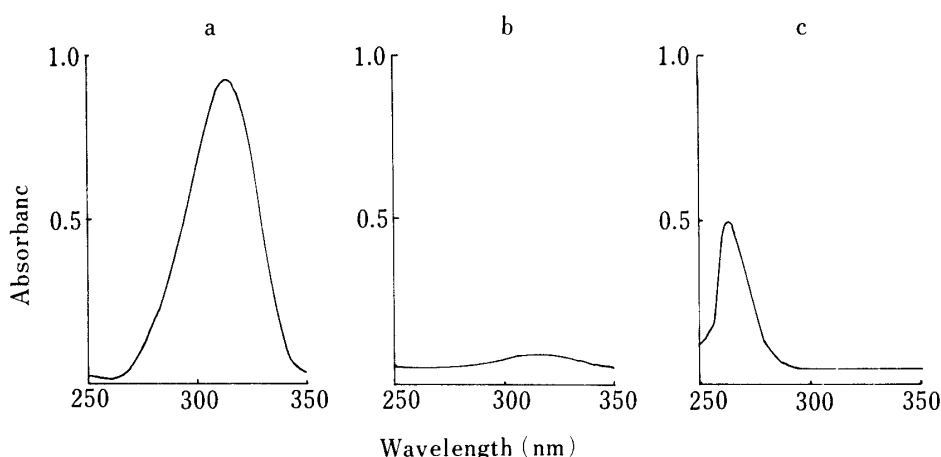


Fig. 8. UV Absorption Spectra of the Hydrolyzed Reaction Product of Clavulanic Acid with Imidazole

Clavulanic acid was reacted with imidazole (a). The solution containing the reaction product was acidified with 1 N HCl (b), and the acidic solution was realkalized with 3 N NaOH solution (c). For detailed procedure, see Chart 1.

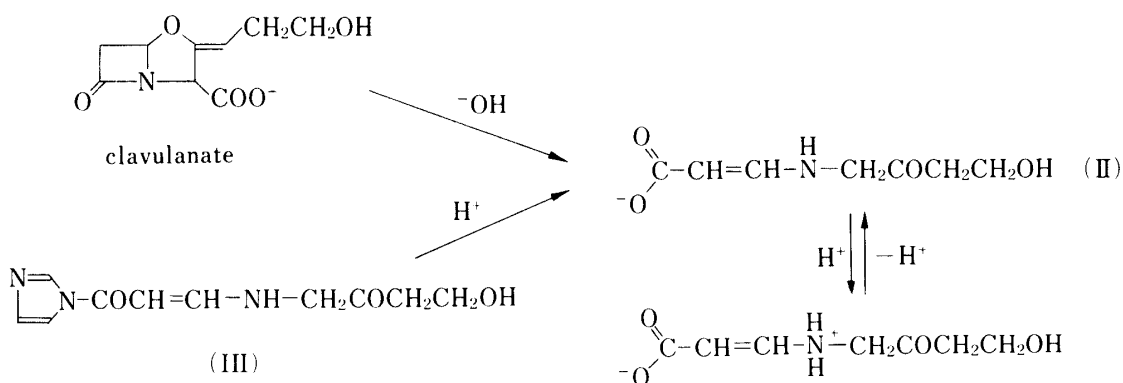


Fig. 9. Alkaline Degradation Pathway of Clavulanic Acid in Aqueous Solutions

Fig. 7b was realkalized with NaOH solution to pH 13.5, the UV absorption around 260 nm reappeared (Fig. 7c). The reappearance of the absorption spectrum was confirmed by the fact that two-fold dilution of the solution of Fig. 7a resulted in a decrease of the absorbance by half (Fig. 7c). The pH dependence of UV absorbance of the degradation product after realkalization showed a maximum and constant absorbance at pH > 13. These results suggest that the degradation product may suffer acid-base conversion. Bird *et al.*<sup>10)</sup> isolated and elucidated the structure of the reaction product of clavulanic acid with imidazole as III, which has a UV absorption maximum at 312 nm. This reaction involves the attack of imidazole on the  $\beta$ -lactam ring followed by fission of the C(5)–O(4) bond and decarboxylation at the 2-position of the oxapenam ring. We reconfirmed this reaction and subjected III to further degradation; when the solution containing III (Fig. 8a) was acidified with HCl to pH 0.75, the UV absorption between 270 and 340 nm almost disappeared (Fig. 8b). Subsequent realkalization of this solution to pH 13.6 by addition of NaOH solution produced UV absorption with  $\lambda_{\max}$  263 nm (Fig. 8c). When the concentration of imidazole was decreased, the  $\lambda_{\max}$  value (263 nm) moved to around 260 nm, which corresponds to  $\lambda_{\max}$  observed in the degradation of potassium clavulanate in alkaline aqueous solution as mentioned above. This demonstrates the hydrolytic elimination of the imidazole group from III in acidic solution (Fig. 9). Davies and Howarth<sup>11)</sup> reported that I is produced by methanolysis of sodium



clavulanate. As mentioned above, we also found that I has strong UV absorption at 268 nm, but its protonated form has almost no UV absorption at 268 nm. These results suggest that the structure of the alkaline degradation product exhibiting UV absorption around 260 nm may be 8-hydroxy-6-oxo-4-aza-2-octenoic acid (II), and that its protonated form may have no UV absorption around 260 nm (Fig. 7). Thus, the disappearance and reappearance of UV absorption under acidic and alkaline conditions may be related to protonation and deprotonation on the amino group. However, the intramolecular condensation product corresponding to IV was not formed by the degradation in alkaline aqueous solutions.

#### HPLC Determination of Clavulanic Acid by Post-Column Alkaline Degradation

It was found that potassium clavulanate is degraded in NaOH-saturated methanol to yield IV *via* I, and in aqueous solution (pH 13) to yield II, and that I has maximum UV absorption at 268 nm, IV at 231 nm, and II around 260 nm. In the previous paper,<sup>15)</sup> we reported that the HPLC separation of clavulanic acid from ordinary urinary components can be attained by a reversed-phase system using TBAB as an ion-pairing agent. Thus, we tried to incorporate this alkaline degradation as a post-column stage of an HPLC determination of clavulanic acid in plasma and urine. In applying the alkaline degradation as the HPLC detection system, it was necessary to confirm that a similar reaction to yield product(s) suitable for UV detection occurs in the post-column reactor where the fraction containing clavulanic acid in the mobile phase is mixed with aqueous NaOH solution, and also to check that such a mobile phase giving a high UV response could also provide good separation. Thus, we examined the effect of organic modifiers (methanol and acetonitrile) of the mobile phase on the post-column reaction and retention of clavulanic acid.

Figure 10a shows the UV absorption spectra of potassium clavulanate in 5 mM TBAB + 1 mM  $\text{Na}_2\text{HPO}_4$  + 1 mM  $\text{NaH}_2\text{PO}_4$ /CH<sub>3</sub>CN = 5/1 (v/v) at 0, 1, 5, and 10 min after addition of NaOH solution (final pH 13.1). The  $\lambda_{\text{max}}$  was around 260 nm, and the maximum UV absorbance was attained at 5 min after addition of NaOH solution. This spectral change was similar to that occurring on alkaline degradation of potassium clavulanate in aqueous NaOH solution (Fig. 6). Figure 10b shows the UV-absorption spectra of potassium

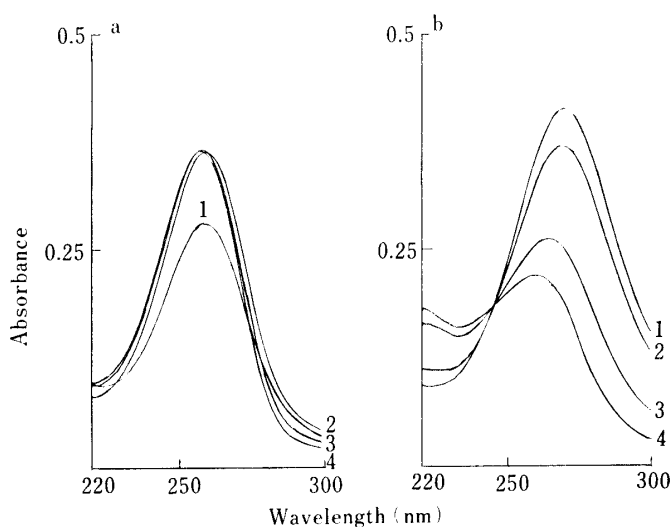


Fig. 10. UV Absorption Spectra of Clavulanic Acid Dissolved in Alkaline Mobile Phase

a: Solvent, 5 mM TBAB + 1 mM  $\text{Na}_2\text{HPO}_4$  + 1 mM  $\text{NaH}_2\text{PO}_4$ /CH<sub>3</sub>CN = 5/1 (v/v). b: Solvent, 5 mM TBAB + 1 mM  $\text{Na}_2\text{HPO}_4$  + 1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH = 5/1 (v/v). A 2.4 ml aliquot of each clavulanic acid solution (7.00  $\mu\text{g}/\text{ml}$ ) was mixed with 1.2 ml of 0.5 N NaOH solution. Reaction time: 1, 0 min (immediately after addition of NaOH solution); 2, 1 min; 3, 5 min; 4, 10 min.

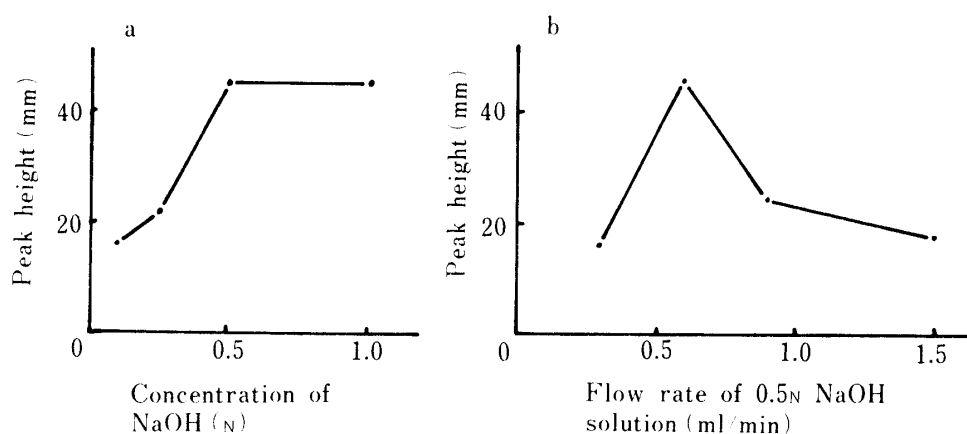


Fig. 11. Examination of Post-Column Reaction Conditions

A 5  $\mu$ l portion of clavulanic acid solution (16.0  $\mu$ g/ml) was injected into the chromatograph under the conditions for analysis of urine samples (see Experimental). Sensitivity: 0.04 aufs. a: The concentration of NaOH solution was varied from 0.1 to 1.0 N with a constant flow rate of 0.6 ml/min. b: The flow rate was varied from 0.3 to 1.5 ml/min with a constant concentration of 0.5 N.

clavulanate in 5 mM TBAB + 1 mM  $\text{Na}_2\text{HPO}_4$  + 1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH = 5/1 (v/v) with NaOH solution (final pH 13.2), where the spectra were recorded at reaction times of 0, 1, 5, and 10 min. It was found that the maximum UV absorbance with a broad absorption band at  $\lambda_{\text{max}}$  270 nm was obtained just after (0 min) addition of NaOH solution, and that as the reaction proceeded,  $\lambda_{\text{max}}$  of the solution shifted from 270 to 260 nm with a concomitant decrease in the intensity at  $\lambda_{\text{max}}$ . This spectral change was similar to that seen in the degradation of potassium clavulanate in NaOH-saturated methanol (Fig. 2). It is generally desirable for the post-column reaction to proceed rapidly and to afford a product which gives a high and reproducible detector response. The results in Fig. 10 show that methanol provides a faster and higher UV response than acetonitrile and that the change in absorbance during the initial couple of seconds of reaction time is smaller in methanol than acetonitrile. Thus, methanol is preferable to acetonitrile as the mobile phase of HPLC for the present purpose. We examined various factors affecting the retention and separation of clavulanic acid, and finally concluded that the following mobile phase system was suitable for HPLC analysis of clavulanic acid; 5 mM TBAB + 1 mM  $\text{Na}_2\text{HPO}_4$  + 1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH = 5/1 (v/v) for urine samples, and 5 mM TBAB + 0.1 mM  $\text{Na}_2\text{HPO}_4$  + 0.1 mM  $\text{NaH}_2\text{PO}_4$ /MeOH = 3/1 (v/v) for plasma samples. The detection wavelength was fixed at 270 nm, because this coincides with  $\lambda_{\text{max}}$  observed in alkaline degradation of potassium clavulanate in the mobile phase system for plasma and urine samples. Although a complicated degradation reaction occurred in such a solution as described above, I seems responsible for the UV absorption at 270 nm.

Next, the flow rate and concentration of NaOH solution was examined in an actual detection system, where the flow rate of mobile phase was fixed at 1.2 ml/min. The effects of flow rate and concentration of NaOH solution on the UV absorbance at 270 nm are shown in Fig. 11. When the concentration was increased from 0.1 to 1 N at a constant flow rate (0.6 ml/min), the chromatographic intensity (peak height) for a given amount of clavulanic acid reached a maximum in the region above 0.5 N (Fig. 11a) where the pH of the reaction solution was 13.1. When the flow rate of NaOH solution was varied between 0.3 and 1.5 ml/min at a constant concentration (0.5 N), the peak height exhibited a maximum at 0.6 ml/min (Fig. 11b). The effect of reaction time was examined by delivering 0.5 N NaOH solution at 0.6 ml/min to reaction coils of various lengths between 0.5 and 4 m (0.25 mm i.d.). Although a constant peak height was obtained regardless of the length, it was advantageous to use a rather long coil in order to reduce the noise level. The actual reaction time was less

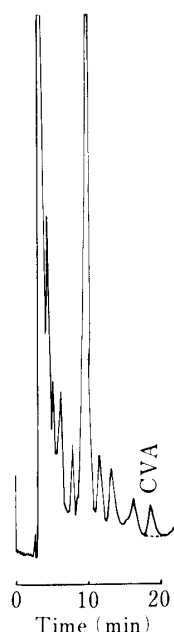


Fig. 12. Separation of Clavulanic Acid from Ordinary Urinary Components

Concentration of clavulanic acid,  $4.00 \mu\text{g/ml}$ ; injection volume,  $5 \mu\text{l}$ ; sensitivity, 0.04 aufs. The dotted line indicates urine blank. CVA: clavulanic acid.

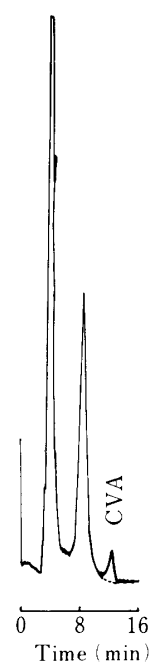


Fig. 13. Separation of Clavulanic Acid from Ordinary Plasma Components

Concentration of clavulanic acid,  $0.26 \mu\text{g/ml}$ ; injection volume,  $50 \mu\text{l}$ ; sensitivity, 0.01 aufs. The dotted line indicates plasma blank. CVA, clavulanic acid.

TABLE I. Recovery (Mean  $\pm$  S.D.) of Clavulanic Acid from Urine and Plasma

	Added ( $\mu\text{g/ml}$ )	Calculated ( $\mu\text{g/ml}$ )	Recovery (%)
Urine	32.0	$31.8 \pm 0.45$	$99.3 \pm 1.41$
	4.00	$4.24 \pm 0.05$	$106.0 \pm 1.25$
Plasma	2.10	$2.07 \pm 0.06$	$98.6 \pm 2.86$
	0.53	$0.50 \pm 0.03$	$94.3 \pm 5.66$

( $N=3$ )

than 5 s, when a 2 m coil was used.

Under the optimum conditions thus established, a  $5 \mu\text{l}$  portion of control urine spiked with clavulanic acid ( $4.00 \mu\text{g/ml}$ ) was injected into the chromatograph. Figure 12 shows the resultant chromatogram, indicating the separation of clavulanic acid from ordinary urinary components. Figure 13 demonstrates the separation of clavulanic acid from ordinary plasma components. The intensities and retention times of background peaks due to urine and plasma components were not affected by addition of NaOH solution for the post-column reaction. The limit of accurate determination was as low as  $0.1 \mu\text{g/ml}$  at maximum sensitivity with 50 and  $25 \mu\text{l}$  aliquots of plasma and urine samples, respectively. The calibration plots, which were obtained by plotting peak height vs. concentration, were linear over the ranges of 0.2–20 and 0.5–500  $\mu\text{g/ml}$  for plasma and urine samples, respectively, and passed through the origin when extrapolated. Table I shows the recoveries of clavulanic acid from spiked urine and plasma, and the coefficients of variation. The precision was of the order of 1.41 and 1.25% for urine sample at levels of 32.0 and  $4.00 \mu\text{g/ml}$ , respectively, and of 2.86 and 5.66% for plasma sample at levels of 2.1 and  $0.53 \mu\text{g/ml}$ , respectively. In order to protect the instrument from

strong alkali, the flow system, especially reagent-delivering pump, post-column reaction coil, and detection flow-through cell, should be thoroughly cleaned with dilute nitric acid and water after use. With this precaution, the present method permits reproducible assays of clavulanic acid in plasma and urine at concentrations as low as 0.1  $\mu\text{g/ml}$  with minimum pretreatment. This method should be applicable to the determination of clavulanic acid in plasma and urine after oral administration of a combined dose of clavulanic acid and amoxicillin.

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