HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLAVULANIC ACID IN HUMAN URINE

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An ion pair reversed phase HPLC method for the determination of clavulanic acid has been developed. Since clavulanic acid had poor absorption (λ_{max} 201 nm in water) in a UV-region suitable for HPLC detection, the detectability was enhanced by bathochromic shift of λ_{max} due to solvent effect. The shifts of λ_{max} were measured with the solutions containing clavulanic acid, tetrabutylammonium bromide, and phosphate buffer salts in aqueous methanol. The magnitudes of the observed shifts were investigated with respect to pH, ionic strength, methanol content, and TBAB concentration. The results indicated that TBAB concentration was the predominant factor responsible for the bathochromic shifts. Taking account of the results together with solvent effects on the retention of clavulanic acid on hydrophobic stationary phase, HPLC condition suitable for detection and separation of clavulanic acid in urine was established as follows; mobile phase: 10 mM TBAB+0.6 mM NaH_2PO_4 +0.4 mM Na_2HPO_4 in H_2O-MeOH, 10: 1 (v/v) (pH 7.02), flow rate: 1.5 ml/minute, stationary phase: LiChrosorb RP-18 (25 cm×4.6 mm i.d.), detection: UV 220 nm. The applicability of the present method is demonstrated by determining the time course of urinary excretion of clavulanic acid after oral administration of a conjugated tablet of clavulanic acid and amoxicillin to human subject.

Clavulanic acid, a novel fused β-lactam produced by *Streptomyces clavuligerus* ATCC 27064, is known as a potent inhibitor against β-lactamases from a wide range of Gram-positive and Gram-negative bacteria.[1~5] This drug is, therefore, anticipated to potentiate the activity of β-lactam antibiotics inactivated by β-lactamase-producing bacteria. A number of *in vitro* microbial investigations have revealed that clavulanic acid used in combination with certain penicillins or cephalosporins exhibits marked reduction in MIC values against various β-lactamase-producing clinical isolates.[6~9] The *in vivo* studies on this drug, however, appeared in a limited number of literatures: GOLDSTEIN *et al.*[10] found that the conjugated dose of clavulanic acid and amoxicillin was able to clean the turbid urine of patients with amoxicillin-resistant urinary tract infections, and HEEREMA *et al.*[11] reported a similar observation with respect to the mice with renal infections. Some information also has been published on the chemical properties of clavulanic acid; the available papers described the chemical[12] and kinetic[13] investigations of the inactivation of β-lactamase and biosynthesis of clavulanic acid.[14,15]

The assay method so far employed in the previous works has been limited to microbiological methods. None referred to chemical and spectrometric procedures for the assays of clavulanic acid, possibly because clavulanic acid has no appreciable absorption in a UV region above 210 nm,[16] and neither iodometric nor hydroxamate assays are suitable for quantitative purposes.[14]

The present work attempts to investigate solvent effects on λ_{max} shifts of clavulanic acid solutions, to find a solvent system appropriate to UV detection and separation by a reversed phase HPLC, and to demonstrate the applicability of the established method to the determination of clavulanic acid excreted in human urine.

Experimental

Reagents and Materials

Clavulanic acid (potassium salt) and conjugated tablet of clavulanic acid and amoxicillin (BRL 25000) are gifts from Beecham Yakuhin Co. Ltd. (Tokyo, Japan). Tetrabutylammonium bromide (TBAB) and buffer salts of reagent grade were obtained from Nakarai Chemicals, Ltd. Deionized distilled water was used for the preparations of solutions for the measurements of UV-spectra and mobile phase of HPLC.

Chromatography

A high performance liquid chromatograph (Twinkle, Jasco, Tokyo, Japan) equipped with a variable wavelength UV-detector (UVIDEC-100-III, Jasco) was used with a stationary phase of LiChrosorb RP-18 (E. Merck, West Germany) packed in a 25 cm×4.6 mm i.d. stainless steel tubing, and a mobile phase of MeOH - H_2O containing various concentrations of TBAB and phosphate buffer salts ($NaH_2PO_4+Na_2HPO_4$), whose flow rate was maintained at 1.5 ml/minute (60 kg/cm^2). A short precolumn (5 cm×4.6 mm i.d.) packed with LiChrosorb RP-2 was used to guard the main column and all operations were carried out under ambient condition.

UV Spectra

A known amount of clavulanic acid was dissolved in various solvents to make final concentrations at about 5×10^{-4} M, and UV spectra between 200 and 260 nm were measured on a UVIDEC 505 Spectrophotometer (Jasco). The solvent composition was varied with respect to (1) methanol content, (2) pH, (3) ionic strength (μ), and (4) TBAB concentration as follows; (1) MeOH - H_2O=0~7: 3 (v/v) containing 0.5 mM TBAB+16 mM NaH_2PO_4+4 mM Na_2HPO_4, pH 6.38~8.07, (2) MeOH - H_2O= 1: 4 (v/v) containing 0.5 mM TBAB+phosphate buffer salts, μ=0.02~0.03, pH 5.02~9.01, (3) MeOH - H_2O=1: 4 (v/v) containing 0.5 mM TBAB+phosphate buffer salts, μ=0.003~0.337, pH 6.28~6.74, (4) MeOH - H_2O=1: 4 (v/v) containing 0 to 10 mM TBAB+16 mM NaH_2PO_4+4 mM Na_2HPO_4, pH 6.72~6.77. In the conditions (2) and (3), the values of ionic strength (μ) are given as equivalent to those in water solution. These solvents were also used as reference solutions for the measurements of UV spectra.

Results and Discussion

The first target of the present investigation was to find out a solvent system suitable for a reversed phase HPLC analysis of clavulanic acid. However, since it has been known that clavulanic acid has no appreciable absorption in a UV-region easily accessible to HPLC detection,[14,16] we tried to

Fig. 1. Dependence of λ_{max} on methanol content.

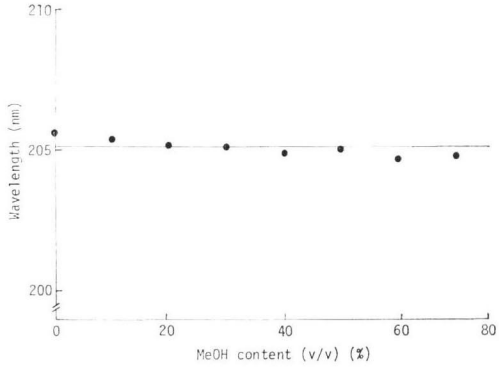


Fig. 2. Dependence of λ_{max} on pH.

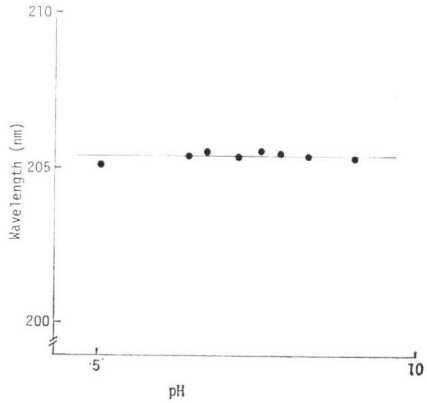


Fig. 3. Dependence of λ_{max} on ionic strength.

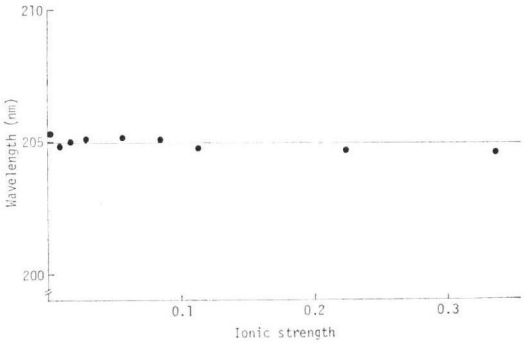
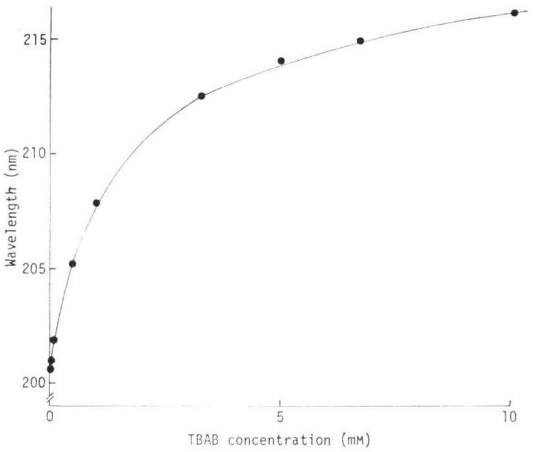


Fig. 4. Dependence of λ_{max} on TBAB concentration.



enhance the detectability with the solvent effect on the bathochromic shift of λ_{max}, taking concomitant effect on retention behavior into account. This is why the solvent systems involved the variations of pH, ionic strength, methanol content and concentration of TBAB which is also expected to affect the retention of clavulanic acid by ion pair formation.

The dependence of λ_{max} on the methanol contents observed under the condition (1) is given in Fig. 1, where it is found that λ_{max} suffered almost no change against the increasing concentration of methanol up to 70%, and a very slight decrease of λ_{max} was accompanied by the 10 times increase of buffer concentration. The use of ethanol instead of methanol in the solvent system (1) gave similar results. Fig. 2 depicts λ_{max} *vs.* pH profile obtained by the condition (2), indicating that there is no apparent dependence on pH between 5.0 and 9.0. The relationship between λ_{max} and ionic strength observed under the condition (3) is illustrated in Fig. 3, which exhibits that increasing ionic strength up to 0.337 exerts almost no effect on the change in λ_{max}. In contrast with these results, the TBAB concentration was found significantly responsible for the bathochromic shift of λ_{max}. As shown in Fig. 4, the value of λ_{max} which was 201 nm in the absence of TBAB and also in pure water increased rapidly with initial increase in the TBAB concentration followed by gradual approach to about 216 nm at 10 mM. Thus it follows that the constant shifts of λ_{max} to about 205 nm shown in Figs. 1, 2, and 3 arise obviously from the presence of 0.5 mM TBAB in the solvent systems of (1), (2), and (3) because the shift at corresponding TBAB concentration found in Fig. 4 is almost to the same extent. These results suggest that the use of TBAB-containing solvent system as a mobile phase of HPLC can afford the detection of clavulanic acid at an accessible UV-wave length.

In establishing HPLC condition, it was naturally noticed that the above mentioned factors, pH, ionic strength, methanol content, and concentration of TBAB, should also exert significant effects on the retention of clavulanic acid on the hydrophobic stationary phase. In order to find out the compromising condition, the effect of TBAB concentration on the retention of clavulanic acid was investigated, while other conditions could be selected without regard to detection wavelength. Fig. 5 illustrates the dependence of capacity factor of clavulanic acid on the concentration of TBAB, where the concentration was varied to cover the range shown in Fig. 4. The observed profile exhibits a curve passing through a maximal point as often seen in ion pair reversed phase liquid chromatography

Fig. 5. Dependence of capacity factor of clavulanic acid on TBAB concentration.

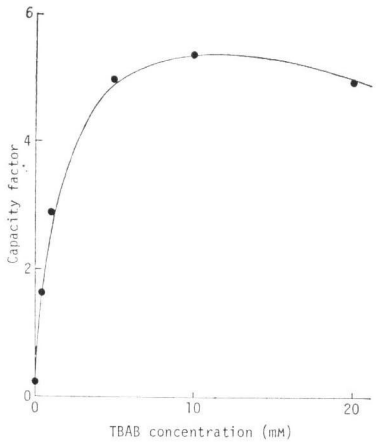


Fig. 6. Chromatographic peak height for a constant amount of clavulanic acid at various detection wavelengths.

Injection volume: 10 μl of 600 μg/ml aqueous solution, Sensitivity: 0.16 aufs, Mobile phase: H_2O-MeOH=5: 1 (v/v) containing 5 mM TBAB+ 0.4 mM Na_2HPO_4+0.6 mM NaH_2PO_4 (pH 7.25), Other coditions: see Experimental part.

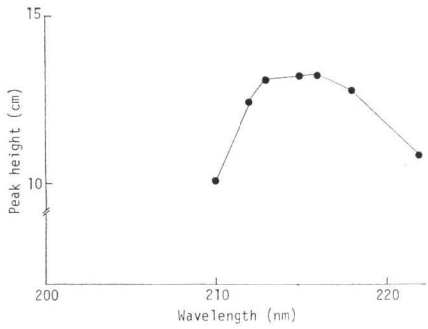
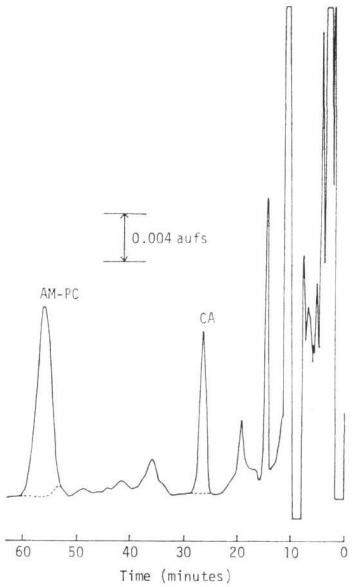


Fig. 7. Chromatograms of human urine excreted at 3 hours after oral administration of a conjugated tablet of clavulanic acid (125 mg) and amoxicillin (250 mg).

CA: clavulanic acid, AM-PC: amoxicillin, Injection volume: 20 μl, HPLC conditions: see Experimental part.



of ionizable organic substances. Thus, it is found that detectability and retention of clavulanic acid are flexible with respect to TBAB concentration between 0 and 10 mM.

In line with the main object of the present work, we examined the various conditions of these factors and finally obtained the following mobile phase system suitable for HPLC analysis of clavulanic acid in human urine; aqueous 10 mM TBAB+0.6 mM NaH_2PO_4+0.4 mM Na_2HPO_4 solution - MeOH=10: 1 (v/v) (other conditions, see Experimental). The actual detectability of clavulanic acid in this solvent system was investigated by changing detection wavelength between 210 to 222 nm. Fig. 6 shows the change in the chromatographic intensity (peak height) for a given amount of clavulanic acid against detection wavelength. It is found that maximal and constant response can be attained at a wavelength between 213 and 217 nm. However, the detection at 220 nm was rather advantageous in lowering the intensities of interfering peaks due to endogenous urinary components. Fig. 7 shows the consequent chromatogram of urine excreted at 3 hours after oral administration of a conjugated tablet of clavulanic acid (125 mg) and amoxicillin (250 mg) to a human subject, where a 20 μl portion of neat urine passed through a 0.45 μm pore size membrane filter was injected. The peaks of unchanged clavulanic acid and amoxicillin were well separated from the background peaks, and their concentrations

Table 1. Urinary excretion amounts of clavulanic acid (mg) after oral administration of a conjugated tablet containing 125 mg clavulanic acid and 250 mg amoxicillin.

Time (hour)	T.N.	J.H.	M.M.	Mean	S.D.
0 ~0.5	0.19	0.28	1.50	0.66	0.60
0.5~1.0	15.67	3.16	8.49	9.11	5.13
1.0~1.5	11.99	14.69	10.98	12.55	1.57
1.5~2.0	8.25	15.66	10.07	11.33	3.15
2.0~2.5	4.37	11.61	6.71	7.56	3.02
2.5~3.0	3.19	7.66	4.30	5.05	1.90
3.0~4.0	3.49	7.20	5.30	5.33	1.51
4.0~5.0	1.28	3.28	1.73	2.10	0.86
5.0~6.0	0.85	1.07	1.04	0.99	0.10
6.0~8.0	0.69	0.45	1.20	0.78	0.31
Total	49.97	65.06	51.32	55.45	6.82

Table 2. Urinary excretion amounts of amoxicillin (mg) after oral administration of a conjugated tablet containing 125 mg clavulanic acid and 250 mg amoxicillin.

Time (hour)	T.N.	J.H.	M.M.	Mean	S.D.
0 ~0.5	1.54	0.35	5.69	2.53	2.29
0.5~1.0	30.71	3.12	21.50	18.44	11.47
1.0~1.5	33.80	24.91	22.22	26.98	4.95
1.5~2.0	20.36	40.33	20.74	27.14	9.33
2.0~2.5	13.51	32.67	19.40	21.86	8.01
2.5~3.0	9.54	22.76	16.58	16.29	5.40
3.0~4.0	10.51	17.92	20.04	16.16	4.09
4.0~5.0	4.20	8.32	8.65	7.06	2.02
5.0~6.0	2.57	3.83	3.45	3.28	0.53
6.0~8.0	2.54	1.31	4.00	2.62	1.10
Total	129.28	155.52	142.27	142.39	10.67

Fig. 8. Time courses of urinary excretion rates of clavulanic acid and amoxicillin following oral administration of a conjugated tablet containing 125 mg clavulanic acid and 250 mg amoxicillin.

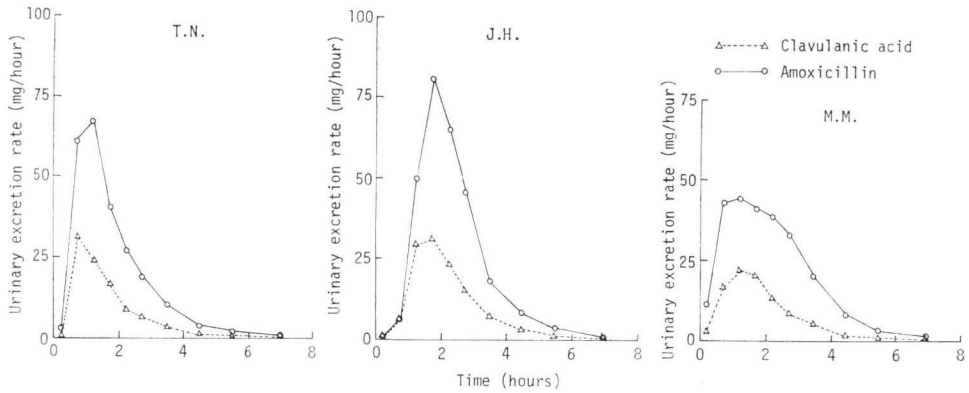


Table 3. Statistical moments and rate constants for clavulanic acid and amoxicillin.

Parameter	T.N.	J.H.	M.M.	Mean	S.D.
Clavulanic acid					
X^{∞} (mg)	50.19	65.15	51.66	55.67	6.733
MRT (hour)	1.781	2.229	2.104	2.038	0.1887
k_a (hour^{-1})	2.153	1.794	1.417	1.788	0.3005
k_e (hour^{-1})	1.391	0.9763	0.8631	1.077	0.2269
Amoxicillin					
X^{∞} (mg)	130.0	155.8	143.5	143.1	10.54
MRT (hour)	1.943	2.396	2.361	2.233	0.2058
k_a (hour^{-1})	1.866	1.385	1.109	1.453	0.3128
k_e (hour^{-1})	1.113	0.8969	0.7026	0.9042	0.1676

were estimated to be 180 μg/ml and 530 μg/ml, respectively. The detection limit of clavulanic acid under the present condition was about 5 μg/ml when 20 μl of urine was injected.

The urinary excretion amounts of intact clavulanic acid and amoxicillin following oral administration of a conjugated tablet to three healthy male subjects were thus determined by referring to the calibration graphs obtained by plotting peak height *vs.* known concentration of respective substances. The results are listed in Tables 1 and 2. The excretion rate *vs.* time curves obtained from the data in Tables 1 and 2 are shown in Fig. 8. From these results, the values for excretion amount at infinite time (X^{∞}) and mean residence time (MRT) were claculated according to moment analysis method[17], and absorption and excretion rate constants were estimated using linear one compartment model by means of non-linear least squares method. The results are given in Table 3, where the excretions of penicilloic and penamaldic acids are not taken into account, although they are known as the urinary metabolites of amoxicillin in man[18]. The detailed pharmacokinetic consideration on urinary excretions of clavulanic acid, amoxicillin and the latter's metabolites will be given in a subsequent paper.

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