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Fast Atom Bombardment Mass Spectrometry of Isoxazolylpenicillins and Their Metabolites in Man

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Fast atom bombardment mass spectrometry has been applied to the investigation of isoxazolylpenicillins and their metabolites isolated from human urine. All the substances gave positive pseudomolecular ions, as either proton adducts or sodium ion adducts, and some structurally significant fragment ions. The results indicated that the active metabolite and a newly found metabolite are the 5-hydroxymethyl derivative of the parent penicillin and its penicilloic acid, respectively.

Keywords——oxacillin; cloxacillin; dicloxacillin; flucloxacillin; metabolism; urinary excretion; fast atom bombardment mass spectrometry; active metabolite; penicilloic acid; isoxazolylpenicillin

Fast atom bombardment mass spectrometry (FAB-MS) is a new technique[1] that has been successfully used to obtain the molecular weights of and structural information on polar, involatile, thermally labile, and yet biologically important compounds such as peptides,[2] phospholipids,[3] nucleic acid compounds[4] and antibiotics[5,6] which are difficult to analyze by means of the conventional ionization methods. Owing to such difficulties, mass spectrometric investigations of β-lactam antibiotics (penicillins and cephalosporins) have so far been carried out by electron impact or chemical ionization of their various esters, which mainly afforded structurally important fragmentation information. Recently the FAB-MS technique has been applied to the investigation of some penicillins as free acids or alkali metal salts, giving molecular weight information through the formation of pseudomolecular ions.[6] However, a literature survey indicated that there has been no report dealing with the application of FAB-MS to the investigation of drug metabolism in man.

Oxacillin (OX), cloxacillin (CX), dicloxacillin (DX), and flucloxacillin (FX) are the isoxazolyl analogues of semisynthetic penicillins which exhibit broad-spectrum antimicrobial activities[7] along with β-lactamase inhibitory activities.[8] These penicillins are biotransformed in man into penicilloic acids and active metabolites, of which the latter have antimicrobial activity comparable to those of the parent penicillins. Formation of penicilloic acid due to β-lactam ring opening of the parent penicillin is well known to be responsible for the inactivation of most penicillins, while formation of the active metabolite is a feature of isoxazolylpenicillins. The present paper describes a FAB-MS investigation of the intact forms of isoxazolylpenicillins and their metabolites isolated from human urine.

Experimental

Sample Preparations——Sodium salts of oxacillin, dicloxacillin, and flucloxacillin were obtained from Banyu Seiyaku Co. (Tokyo) and sodium cloxacillin was from Meiji Seika Co. (Tokyo). These penicillins were dissolved in

TABLE I. Column Chromatography for the Isolation of Metabolites of Isoxazolylpenicillins[a)]

Samples	Composition of developing solvent (v/v)		
	First run 0.2 M acetate buffer–CH_3CN (pH 5.2)	Second run 0.03 M acetate buffer–CH_3CN (pH 5.6)	Third run H_2O–CH_3CN
OX_I	5:1	20:3	5:1
OX_{II}	5:1	20:3	5:1
OX_{III}	10:1	16:1	5:1
CX_I	40:9	5:1	4:1
CX_{II}	40:9	5:1	4:1
CX_{III}	20:3	8:1	5:1
DX_I	10:3	40:9	4:1
DX_{II}	10:3	40:9	4:1
DX_{III}	40:9	5:1	5:1
FX_I	4:1	40:9	4:1
FX_{II}	4:1	40:9	4:1
FX_{III}	5:1	20:3	5:1

a) The stationary phase was LiChroprep RP-18 (40—63 μm particle diameter). The fraction obtained in each run was analyzed by HPLC.

sulfate buffer solution (pH 2.0) and extracted with ethylene dichloride. Evaporation of the solvent followed by complete drying *in vacuo* gave the free acid of the parent penicillin. The metabolites (penicilloic acid I, active metabolite II, and penicilloic acid of the active metabolite III) were isolated from the urine collected after oral administration of each penicillin to human subjects. The isolations of I and II were achieved by preparative chromatography of neat urine, while III was obtained by the chromatography of ethylene dichloride extracts of the urine. The chromatography was repeated three times on a reversed-phase column (LiChroprep RP-18, 40—63 μm particle diameter, 25 mm i.d. × 31 cm length, E Merck) with developing solvents of various compositions (see Table I). The fraction containing a metabolite to be isolated was collected and concentrated carefully almost to dryness under reduced pressure at 37 °C. The residue was taken up in a small portion of water and subjected to the second chromatographic purification. The fraction obtained was concentrated in the same manner as above, and the residue was dissolved in the sulfate buffer solution and extracted with ethylene dichloride. Evaporation of the solvent gave a white solid, which was again chromatographed on the same column. The metabolite was finally isolated as the free acid, its purity being confirmed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection as described in the previous papers.[9-11]

FAB-MS Measurement——A JMS-DX 300 mass spectrometer equipped with a JMA-3500 data system (JEOL Ltd.) was used for the measurements of FAB mass spectra. Argon was used for the primary beam (3 kV). Spectra were obtained at an accelerating voltage of 3 kV over a mass range from *m/z* 200 to 600. The pressure of argon gas was approximately 5×10^{-5} Torr. The ion source was maintained at ambient temperature. The samples were dissolved in 2 to 3 drops of chloroform, and applied to a stainless steel probe tip with an appropriate amount of glycerol. The probe was immediately inserted into the mass spectrometer. Calibration was done using an electron impact ion source and perfluorokerosene.

Results and Discussion

The positive FAB mass spectra of dicloxacillin and its three metabolites are shown in Fig. 1. The observed mass numbers of pseudomolecular ions and some rational fragment ions of the four isoxazolylpenicillins and their metabolites are listed in Table II. In every case, the protonated molecular ions $[M+H]^+$ were obtained with good intensity. The relative intensities of peaks containing Cl isotope (cloxacillin, dicloxacillin, and flucloxacillin) agree roughly with the expected natural abundances. In addition to $[M+H]^+$, sodium adduct ions $[M+Na]^+$ were also observed in all cases except for flucloxacillin (FX) and the penicilloic

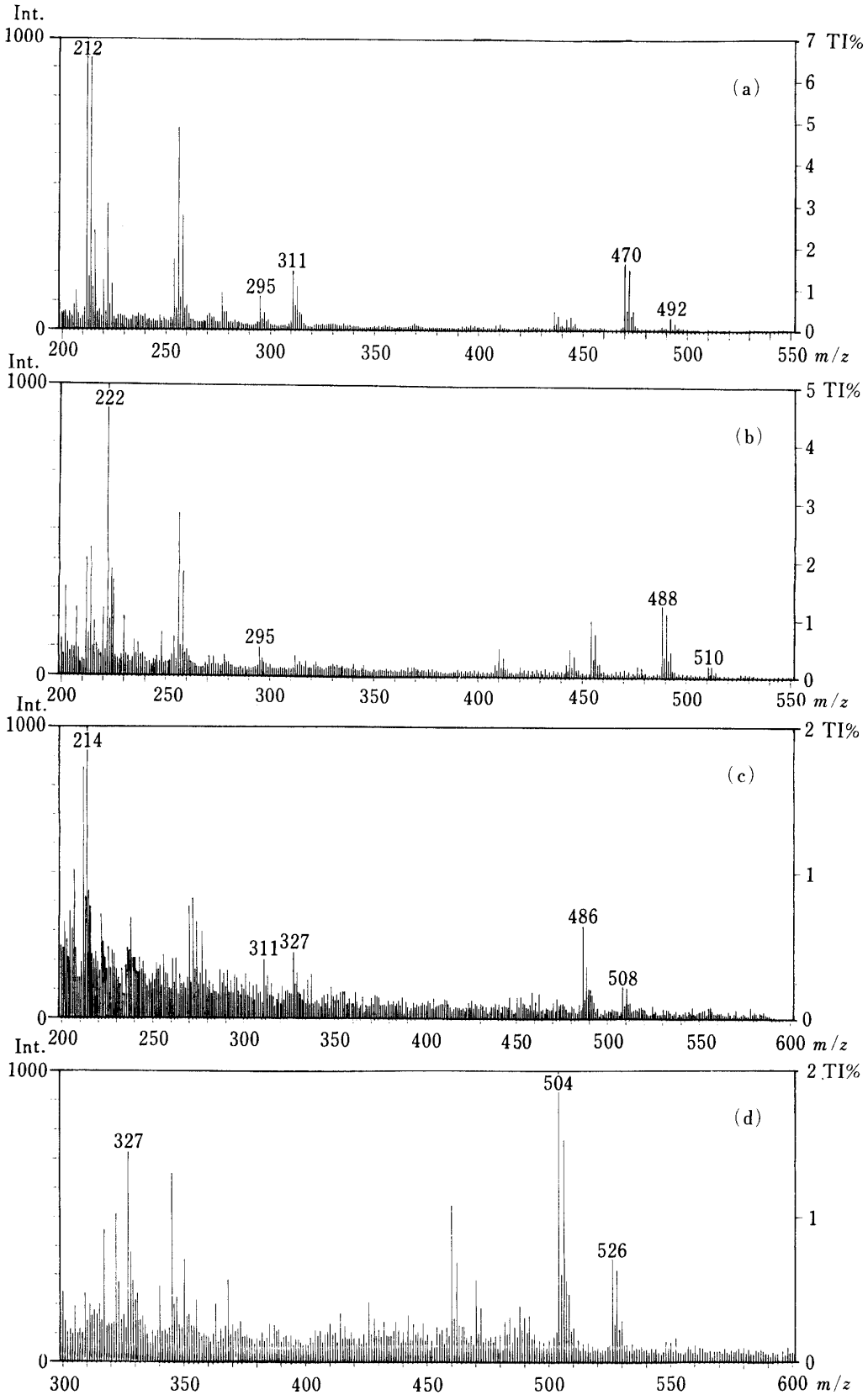

Fig. 1. Positive Ion FAB Mass Spectra of Dicloxacillin and Its Metabolites Excreted in Human Urine

(a) intact form; (b) penicilloic acid (I); (c) active metabolite (II); (d) penicilloic acid of the active metabolite (III).

TABLE II. Observed Mass Numbers (m/z) of Pseudomolecular Ions and Some Fragment Ions of Four Isoxazolylpenicillins and Their Metabolites

	$[M+H]^+$	$[M+Na]^+$	$[F_1+H]^+$	$[F_2+H]^+$
OX	402	424	243	227
OX_I	420	442	—	—
OX_{II}	418	440	259	243
OX_{III}	436	—	—	—
CX	436	458	277	261
CX_I	454	476	—	261
CX_{II}	452	474	293	277
CX_{III}	470	492	—	—
DX	470	492	311	295
DX_I	488	510	—	295
DX_{II}	486	508	327	311
DX_{III}	504	526	—	—
FX	454	—	295	279
FX_I	472	494	—	—
FX_{II}	470	492	311	295
FX_{III}	488	510	—	—

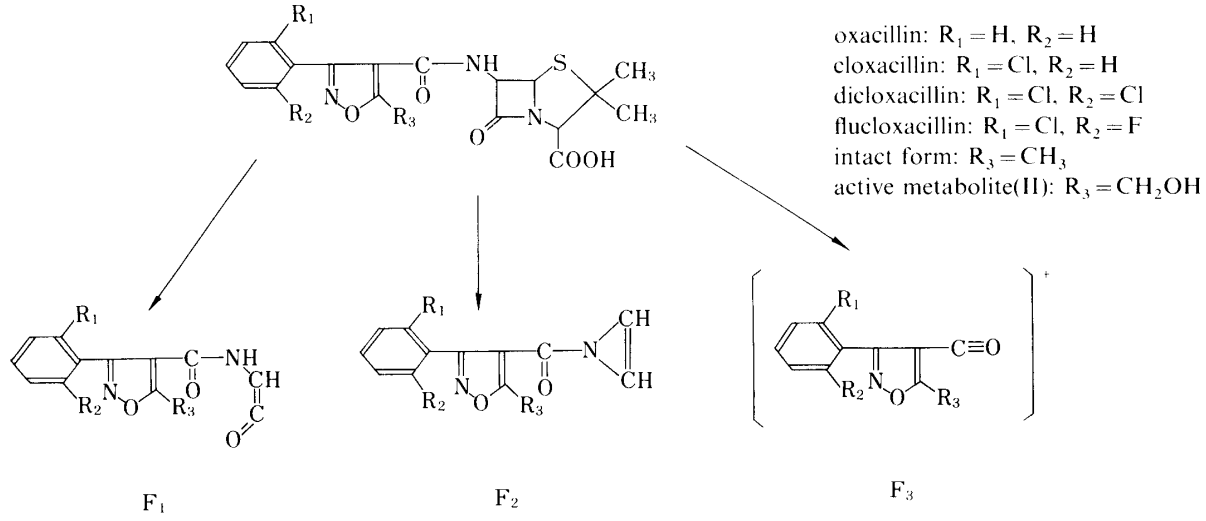

Chart 1. Positive Ion Fragmentation Pathways

acid of the active metabolite of oxacillin (OX_{III}). There are also some additional pseudomolecular ions $[M+2Na-H]^+$ for the active metabolites of oxacillin and flucloxacillin (m/z 462 and 514, respectively). Such alkali metal adduct ions are sometimes observed in the FAB ionization of free acids.[12] There are some fragment ions which are structurally significant. The cleavage of the β-lactam ring yielding a fragment F_1 as depicted in Chart 1 gave the $[F_1+H]^+$ ion for the four isoxazolylpenicillins and their 5-hydroxymethyl metabolites. These fragment ions together with the pseudomolecular ions offer significant information about the lateral chain, which supported the identification of the active metabolite as the 5-hydroxymethyl derivative of the parent penicillin. Another pathway of cleavage of the β-lactam ring yielding fragment F_2 (see chart 1) also gave distinct ions $[F_2+H]^+$ for OX, OX_{II}, CX, CX_I, CX_{II}, DX, DX_I, DX_{II}, FX, and FX_{II}. A similar type of fragmentation has been observed

in the electron impact (EI) mass spectra of methyl esters of isoxazolylpenicillins and their active metabolites isolated from rat urine.[13] In the previous paper,[10] we reported EI mass fragmentography of the trimethylsilyl derivative of the active metabolite of flucloxacillin in man, where a fragment ion corresponding to F_2 was selected as one of the monitoring ions. The fragment F_3, which can be formed by cleavage of the CONH bond in the lateral chain, appeared with very weak or almost no intensity, although Barber *et al.*[6] observed this ion in the FAB mass spectrum of cloxacillin sodium salt.

It follows that the active metabolite was formed by addition of an oxygen atom to the parent penicillin, and subsequent addition of H_2O yielded the newly found metabolite, *i.e.* the penicilloic acid of the active metabolite. In combination with the previous nuclear magnetic resonance spectral result[14] that one proton was eliminated from the methyl group of the parent penicillin, leaving a methylene moiety on the isoxazole ring of the active metabolite, it is concluded that hydroxylation of the 5-methyl group on the isoxazole ring is the process resulting in the formation of the active metabolite.

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References

1) M. Barber, R. S. Bordoli, G. J. Elliott, R. D. Sedgwick and A. N. Tyler, *Anal. Chem.*, **54**, 645A (1982).
2) A. M. Buko, L. R. Phillips and B. A. Fraser, *Biomed. Mass Spectrom.*, **10**, 324 (1983).
3) G. R. Fenwick, J. Eagles and R. Self, *Biomed. Mass Spectrom.*, **10**, 382 (1983).
4) M. E. Rose, C. Longstaff and P. D. G. Dean, *Biomed. Mass Spectrom.*, **10**, 512 (1983).
5) M. Barber, R. S. Bordoli, R. D. Sedgwick, A. N. Tyler and B. W. Bycroft, *Biochem. Biophys. Res. Commun.*, **101**, 632 (1981).
6) M. Barber, R. S. Bordoli, R. D. Sedgwick, A. N. Tyler, B. N. Green, V. C. Parr and J. L. Gower, *Biomed. Mass Spectrom.*, **9**, 11 (1982).
7) R. Sutherland, E. A. P. Croydon and G. N. Rolinson, *Br. Med. J.*, **4**, 455 (1970).
8) J. A. Bach, N. Buono, D. Chisholm, K. E. Price, T. A. Pursiano and A. Gourevitch, *Antimicrob. Agents Chemother.*, **1967**, 328.
9) Y. Murai, T. Nakagawa, K. Yamaoka and T. Uno, *Chem. Pharm. Bull.*, **29**, 3290 (1981).
10) Y. Murai, T. Nakagawa, K. Yamaoka and T. Uno, *Int. J. Pharmaceut.*, **15**, 309 (1983).
11) Y. Murai, T. Nakagawa, K. Yamaoka and T. Uno, *Chem. Pharm. Bull.*, **31**, 3292 (1983).
12) JEOL: FAB-MS Application Data Vol. 106.
13) H. H. W. Thijssen, *J. Antibiot.*, **10**, 1033 (1979).
14) Y. Murai, T. Nakagawa, K. Okumura and T. Uno, *Chem. Pharm. Bull.*, **32**, 3277 (1984).