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Isolation and Identification of Piperacillin Amide as an Impurity in Piperacillin

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Abstract □ Piperacillin amide (IV) was successfully identified as the predominant impurity in commercial lots of piperacillin monohydrate (III). The impurity was isolated *via* a preparative liquid chromatographic scheme utilizing Florisil as the adsorbent and a mobile phase of water–acetonitrile (4:96, v/v). The isolated component had nearly the same reverse-phase HPLC properties as piperacillin and was chemically and thermally unstable. This labile impurity was spectroscopically identified by field desorption (FD), fast atom bombardment (FAB) with collision activation decomposition (CAD), and desorption chemical ionization (DCI) mass spectrometries, and NMR and IR spectrometries. Identity was confirmed on comparison of the chromatographic and spectrometric data of the impurity with an independently synthesized sample of piperacillin amide.

Keyphrases □ Piperacillin amide—impurity in piperacillin, isolation, identification using soft-ionization mass spectra, high-field NMR, and IR spectrometry □ Piperacillin—isolation of an amide impurity by column chromatography, identification using soft-ionization mass spectra, high-field NMR, and IR spectrometry, antibiotic activity

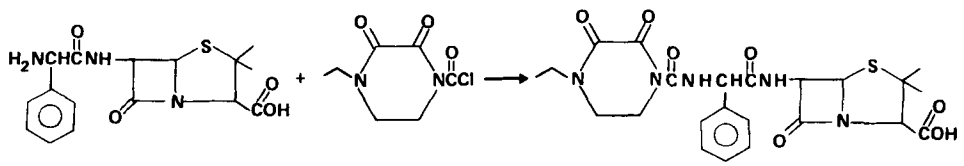
Piperacillin[1] is a third generation semisynthetic penicillin antibiotic with a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative organisms (1). As shown in Scheme I, piperacillin (III) is produced from the reaction of ampicillin (I) with 4-ethyl-2,3-dioxo-1-piperazinecarbonyl chloride (II). Five low-level components, each ≤0.6% w/w, were observed in the final piperacillin product. Two of these components were related to the starting materials: ampicillin and 1-ethyl-2,3-piperazinedione (2). Two components were expected process impurities: the β-lactam hydrolysis product of piperacillin (3) and the condensation product of piperacillin with ampicillin (2). The isolation and identification of the fifth component is the subject of this paper. This component, referred to as the "unknown piperacillin impurity," constituted the major impurity in commercial lots of piperacillin, 0.2–0.4% w/w[2]. This impurity proved to be difficult to isolate chromatographically and to characterize spectrometrically.

Two main factors which complicated the development of a reverse-phase HPLC method for isolating the unknown piperacillin impurity from piperacillin in quantities large enough for spectrometric analysis are:

1. the difficulty in resolving the major impurity from piperacillin because the impurity eluted as a shoulder on the high retention time side of piperacillin;
2. the chemical instability of the unknown material in normal HPLC reverse-phase solvents, *viz.*, methanol and aqueous media, at other than neutral pH for extended periods.

For these reasons, a unique preparative column chromatographic method using an isocratic normal phase was developed which was successful in resolving the unknown piperacillin impurity from piperacillin.

Identification of the isolated impurity was not routine, since it was highly polar and chemically and thermally unstable. The unknown impurity was characterized by mass spectrometry using soft-ionization conditions, *i.e.*, field desorption (FD) (4, 5), fast atom bombardment (FAB) (6, 7), and desorption chemical ionization (DCI) (8, 9). High-field NMR and Fourier transform IR spectrometries were also used to elucidate the structure of the unknown piperacillin impurity. The spectrometric data suggested that the unknown was piperacillin amide (IV). This hypothesis was confirmed by compar-



(I) (II) (III)

Scheme I

[1] Marketed by Lederle Laboratories in the U.S. as Pipracil and in Europe as Pipril.

[2] Penticillin (Toyama Chemicals Co., Ltd. brand of piperacillin marketed in Japan) also contains piperacillin amide, but the quantity is negligible.

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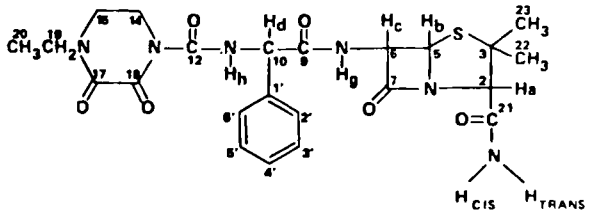
ison of the chromatographic and spectrometric data with that of an authentic piperacillin amide sample prepared in an unambiguous organic synthesis. These results demonstrate that the combination of soft-ionization mass spectral techniques, high-field NMR, and IR are exceptionally effective for identifying small amounts of nonvolatile components.



(IV)

EXPERIMENTAL

Chromatography—A column chromatographic procedure was followed for isolating the unknown piperacillin impurity. The chromatographic column was 80 × 5.2 cm and was packed with 1 kg of Florisil[3], 60–100 mesh. Twenty grams of bulk piperacillin monohydrate were dissolved in 200 mL of acetonitrile and loaded on to the column. The elution solvent consisted of acetonitrile–water (96:4, v/v) with a flow rate of 35 mL/min. The detector was a UV spectrophotometer[4] fitted with a variable-volume flow cell set to monitor at 220 nm. This system operated at ambient temperature. The elution volume of the piperacillin impurity was 2100–4000 mL. This experiment was repeated three times before discarding the column packing. The eluant containing the impurity from three runs was combined, distilled *in vacuo*, and freeze-dried to produce 84 mg of white solid.

Analytical reverse-phase HPLC[5] with UV detection[6] at 220 nm was used for the analysis of bulk piperacillin and the isolated impurity components. A 250 × 4.6-mm column packed with silica gel chemically bonded with octadecylchlorosilane[7] was maintained at 35°C. The mobile phase consisted of methanol–water–0.2 M Na_2HPO_4 (45:34:10, v/v/v) adjusted to pH 6.5 with 0.33 M phosphoric acid. The injection volume of samples was 20 μL, made up in the mobile phase.

Although dimethyl sulfoxide (Me_2SO) is the solvent of choice for the unknown piperacillin impurity, methanol was used even though the impurity underwent a chemical transformation when dissolved in the latter solvent for an extended period (>1 d) at room temperature. At elevated temperatures the reaction rate is enhanced considerably. However, by limiting the contact time to <30 min, this problem was circumvented.

Spectrometry—FD mass spectra were obtained with a double-focusing high-performance mass spectrometer[8]. The FD emitter consisted of a 10-μm tungsten wire with microneedles of pyrolytic carbon grown to a length of ~30 μm. The FD emitter was coated with a Me_2SO solution of the unknown piperacillin impurity sample. On application of the emitter current, very weak signals were observed due to a high sodium ion content contaminating the sample and emitter. The original sample was washed in the following fashion to remove inorganic salts. Approximately 20 mg of the trapped piperacillin impurity was partially dissolved in 5 mL of acetonitrile. On addition of 5 mL of distilled water a white precipitate formed, which was collected on a filter and washed with 1 mL of distilled water. The filtrate was then dried *in vacuo* and analyzed in the FD mode. The sodium signal of the washed sample was reduced considerably, and relatively intense FD signals were produced by the sample. Low-resolution FD data were acquired at a resolution of 1000.

Exact mass measurements in the FD mode were made at a resolution of 6000 in the following fashion. A reference peak in the electron-impact (EI) mode (PFK[9] fragment: $C_{13}F_{19}$, *m/z* 516.9697) was sequentially peak matched against the molecular ion of the unknown piperacillin impurity in the FD mode. The experimental mass difference between the FD and EI modes was measured with a known reference compound peak in both the field ionization (FI) and EI modes [tris(pentafluoroethyl)-*s*-triazine[9]: $C_9F_{15}N_3$, *m/z* 434.9853]. Using this mass difference, the exact mass of the unknown was corrected.

FAB spectra were obtained with a double-focusing high-performance mass spectrometer[8] and with a triple-stage quadrupole mass spectrometer[10]. Collision-activation decomposition (CAD) of selected FAB ions was performed on the latter instrument (10). The fast atom reagent gas was xenon, and the collision gas was argon. The samples for FAB ionization were either mixed directly with glycerol or dissolved in Me_2SO and then mixed with glycerol. FAB spectra of the unknown piperacillin impurity were obtained in both the positive- and negative-ionization modes. FAB/CAD spectra in the positive-ionization mode were obtained on the protonated molecular ion and the sodium adduct molecular ion of the unknown piperacillin impurity and synthetic piperacillin amide (neat and spiked with sodium chloride).

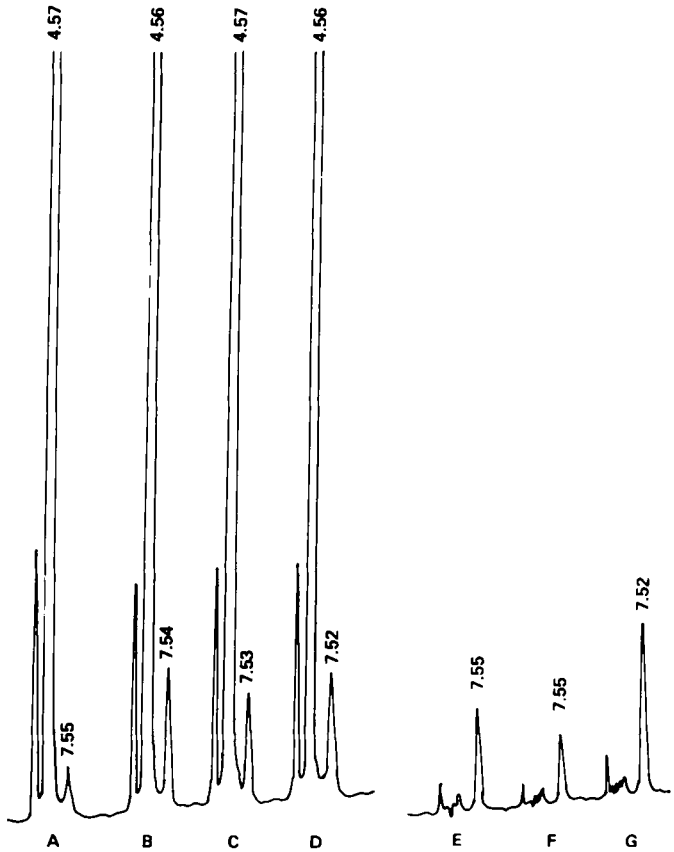


Figure 1—*HPLC chromatograms with retention times (min). Key: (A) bulk piperacillin; (B) bulk piperacillin spiked with unknown piperacillin impurity; (C) bulk piperacillin spiked with synthetic piperacillin amide; (D) bulk piperacillin spiked with unknown piperacillin impurity and synthetic piperacillin amide; (E) unknown piperacillin impurity; (F) synthetic piperacillin amide; (G) unknown piperacillin impurity spiked with synthetic piperacillin amide.*

Desorption chemical ionization (DCI) was performed on a quadrupole mass spectrometer[11] in the positive- and negative-ionization modes with methane and ammonia as the reagent gases. The sample was rubbed onto the surface of one end of a gold wire, ~1 cm long, and the other end was inserted into the tip of the direct insertion probe. This gold wire effectively extended the probe length so that the sample was inserted directly in the ion plasma region of the source. The thermal energy of the ion plasma and rapid heating of the probe gently desorbed the sample from the gold wire surface generating ions of diagnostic interest in the negative-ion mode. The DCI mass spectral data reported in this paper correspond to the first signal in the reconstructed total ion current profile, as the samples were rapidly heated.

The ^1H- and ^{13}C-NMR spectra were obtained on a high-field NMR spectrometer[12] operating in the pulsed Fourier transform mode at 250.13 and 62.89 MHz, respectively. The samples were dissolved in Me_2SO-d_6, and the spectra were referenced to tetramethylsilane. The IR spectra were recorded in 100 scans on a Fourier transform IR system[13] using samples pressed into potassium bromide pellets.

Preparation of Piperacillin Amide—To a solution of isobutyl chloroformate (13.6 g, 100 mmol) in chloroform (100 mL) at −30°C under argon was added a cold solution of piperacillin monohydrate (53.5 g, 100 mmol) in a mixture

[3] Floridin Co., Pittsburgh, Pa.
[4] Model DU; Beckman Instruments, Fullerton, Calif.
[5] Model M6000A HPLC Pump; Waters Associates, Milford, Mass.
[6] Model SF 770 Spectroflow Monitor; Schoeffel Instrument Corp., Westwood, N.J.
[7] Partisil 10/25 ODS; Whatman, Inc., Clifton, N.J.
[8] Model MS-50; Kratos Instrument Corp., Manchester, England.
[9] PCR Research Chemicals, Inc., Gainesville, Fla.
[10] Model TSQ; Finnigan Corp., Sunnyvale, Calif.
[11] Model 4021T and Incos Model 2000 Data System; Finnigan Corp., Sunnyvale, Calif.
[12] Model WM-250; Brucker Instruments Inc., Billerica, Mass.
[13] Model 7199; Nicolet Instrument Corp., Madison Wis.

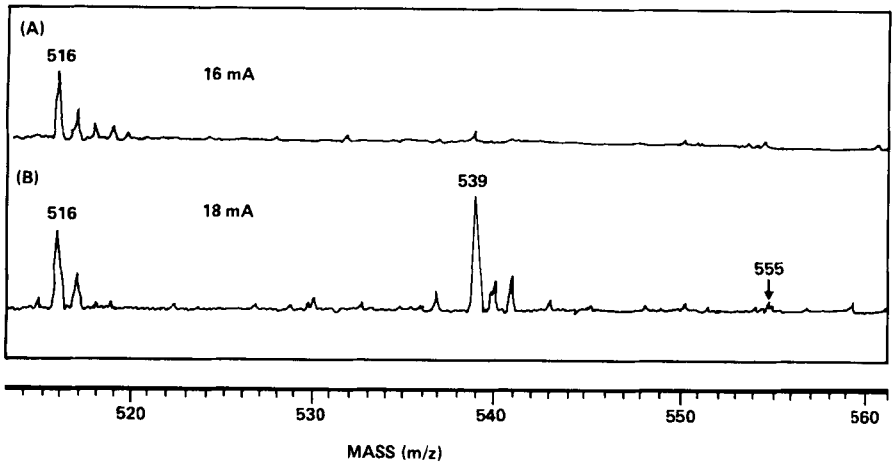


Figure 2—*Molecular ion region of the field desorption mass spectrum of the unknown piperacillin impurity with emitter current at 16 (A) and 18 mA (B).*

of chloroform (500 mL) and triethylamine (10.1 g, 100 mmol, 14 mL) over a 40-min period. The mixture was stirred an additional 1.5 h at 0°C. Ammonia was bubbled through the mixture (a white precipitate formed immediately) in a steady stream for 25 min. The thick white suspension was diluted with 1 L of chloroform and then filtered. The white filter cake was washed with chloroform (2 × 200 mL) and dried *in vacuo* to afford 49 g of crude piperacillin amide, which contained a considerable amount of ammonium chloride.

The aforementioned crude product (30 g) was suspended in 150 mL of distilled water and stirred vigorously for 2 h. The white solid was filtered, washed with acetone (2 × 50 mL) and anhydrous ether (2 × 100 mL), and dried *in vacuo* to afford 24 g of piperacillin amide (78% yield), mp 217–220°C dec[14].

Anal.—Calc. for $C_{23}H_{28}N_6O_6S \cdot 0.2H_2O$: C, 53.13; H, 5.49; N, 16.16; S, 6.17. Found[15]: C, 52.97; H, 5.27; N, 16.12; S, 6.17. KF[16] = 0.66% (which equals $0.2H_2O$).

Spectral Data—All the spectral data listed below were identical for both the unknown piperacillin impurity and the synthetic piperacillin amide unless noted otherwise. FD-MS *m/z* (%) for the piperacillin impurity: 555, 539, 517, 516, 375, 374, 373, 143, 142, and 141; for piperacillin amide: 516(18), 375(18), 359(18), 274(12), 160(8), 159(14), 158(12), 144(20), 143(100), and 142(100). +FAB-MS *m/z* for the piperacillin impurity and amide spiked with sodium chloride: 539, 519, 518, 517, 397, 359, 333, 241, 159, and 143. −FAB-MS *m/z* for the piperacillin impurity: 517, 516, 515, and 373. +FAB/CAD(517)-MS *m/z* (%): 359(13), 274(12), 186(20), 175(3.5), 174(4), 159(100), 143(82), 132(4), 115(5), and 114(6). +FAB/CAD(539)-MS *m/z* (%): 475(11), 397(100), 181(2), and 165(20). +DCI-MS *m/z* (%): 375(15), 159(60), and 143(100). −DCI-MS *m/z* (%); 516(22), 374(100), 341(35), 331(35), 330(45), 233(85), 175(77), 174(62), 173(50), and 155(35). ^{1}H-NMR (Me_2SO-d_6): δ 1.18 (t, NCH_2CH_3, J = 7.5 Hz), 1.39 (s, CH_3), 1.53 (s, CH_3), 3.32 (q, NCH_2CH_3, J = 5.0 Hz), 3.56 (m, CH_2), 3.90 (m, CH_2), 4.10 (s, CH_a), 5.36 (d, CH_b, J = 3.5 Hz), 5.46 (dd, CH_c), 5.72 (d, CH_d, J = 7.0 Hz), 7.2–7.5 (m, 5 ArH; s, NH_{cis}), 7.58 (s, NH_{trans}), 9.35 (d, NH_g, J = 7.0 Hz), and 9.84 ppm (d, NH_h, J = 7.5 Hz). ^{13}C-NMR (Me_2SO-d_6): δ 11.9 (q, C-20), 26.5 (q, C-22), 30.0 (q, C-23), 40.3 (t, C-19), 41.6 (t, C-15), 42.8 (t, C-14), 56.5 (d, C-6), 57.7 (d, C-10), 63.9 (s, C-3), 66.7 (d, C-5), 70.6 (d, C-2), 126.5 (d, C-3′ and C-5′), 127.8 (d, C-4′), 128.3 (d, C-2′ and C-6′), 137.8 (s, C-1′), 151.8 (s, C-12), 155.3 (s, C-18), 159.4 (s, C-17), 168.8 (s, C-7), 169.3 (s, C-9), and 173.4 ppm (s, C-21).

RESULTS AND DISCUSSION

Florisil was chosen as the adsorbent because it almost irreversibly adsorbs acidic materials (11). Since this material should adsorb piperacillin acid irreversibly, it should be possible to elute the more chemically neutral piperacillin impurity selectively. The resolution of the two components on Florisil was accomplished using an elution solvent mixture containing an unusually high water concentration in acetonitrile, *i.e.*, water–acetonitrile (4:96, v/v). The high water content of the elution solvent did not degrade the Florisil adsorption properties for these very polar materials. Only very low levels of piperacillin were detected in the trapped piperacillin impurity sample using the analytical reverse-phase HPLC conditions described in the *Experimental* section.

Bulk piperacillin (monohydrate) and the trapped unknown piperacillin impurity component, when analyzed under the analytical HPLC conditions, produced the chromatograms illustrated in Fig. 1a and e, respectively. The components eluting at 4.57 and 7.55 min correspond to piperacillin and the unknown piperacillin impurity, respectively.

Similarly, the analytical HPLC chromatograms of the synthetic piperacillin amide and piperacillin spiked with the synthetic piperacillin amide are illustrated in Fig. 1f and c, respectively. In addition, experiments were performed in which piperacillin was spiked with the unknown piperacillin impurity (Fig. 1b) or both the unknown impurity and the synthetic piperacillin amide (Fig. 1d). In all cases, the retention times of the synthetic piperacillin amide and the unknown piperacillin impurity either individually or in the spiking experiments were identical, *i.e.*, within the experimental errors associated with the reproducibility of the HPLC experiment.

The mass spectral properties exhibited by the piperacillin impurity, even under soft-ionization conditions, included (*a*) the difficulty in generating intense molecular and fragment ions of long enough duration for accurate measurement and (*b*) the lability of the chemical bond between the piperazine moiety and the rest of the piperacillin molecule. These mass spectral properties of the piperacillin impurity were similar to those of piperacillin.

The FD mass spectra of the washed piperacillin impurity are illustrated in Fig. 2 as a function of increasing emitter current. At 16 mA (Fig. 2a) an intense ion cluster at *m/z* 516 appears, to which is added at 18 mA (Fig. 2b) an intense ion cluster at *m/z* 539 and a weak peak at *m/z* 555. The ion cluster at *m/z* 539 and the weak peak at *m/z* 555 correspond to the sodium and potassium adducts of the molecular ion, respectively, of the unknown piperacillin impurity. This confirms that *m/z* 516 is $M^{+\cdot}$ and not $[M + H]^+$.

The molecular ion of the unknown piperacillin impurity was peak-matched in the FD mode and had an exact mass of 516.1882. This value is within 9.1 millimass units of $C_{23}H_{28}N_6O_6S$ (516.1791), the corresponding elemental formula of piperacillin amide.

The FD mass spectrum of the synthetic piperacillin amide produced a relatively intense molecular ion as well as a number of fragment ions consistent with its structure. No sodium or potassium adduct molecular ions were observed due to the absence of the cations.

The molecular weight of 516 for the unknown piperacillin impurity as well as its identity as piperacillin amide were confirmed by positive and negative FAB ionization and DCI mass spectrometries. The FAB ionization mass spectra of the unknown piperacillin impurity contained the quasimolecular ions $[M + H]^+$, $[M + Na]^+$, and $[M - H]^-$ at *m/z* 517, 539, and 515, respectively. Weak fragment ions were produced as well and often were difficult to distinguish from the glycerol background spectrum. The identity of the impurity with that of piperacillin amide was confirmed on comparison of the nearly identical ions and intensities produced in the FAB/CAD spectra from the *m/z* 517 and 539 ions for the two materials.

Similarly, the positive and negative DCI spectra of the unknown piperacillin impurity and the synthetic piperacillin amide were identical. The positive DCI spectrum obtained no molecular ion and resembled the spectrum produced under electron-impact conditions. The negative DCI spectrum contained a molecular ion, $M^{-\cdot}$, and diagnostic ions characteristic of the molecule.

The ^{1}H-NMR spectrum of the unknown piperacillin impurity is nearly identical to that of piperacillin except for additional absorption observed in the aromatic proton region, which correspond to two additional protons. These two uncoupled nonequivalent protons correspond to the *cis* and *trans* amide protons of piperacillin amide. Synthetic piperacillin amide gave a NMR spectrum identical with that of the unknown impurity with the exception of the *trans* amide proton chemical shift, 7.58 ppm in piperacillin amide *versus*

[14] Melting points were determined on a Melt-Temp Melting Point Apparatus.

[15] Micro analytical elemental analyses were performed on a Model 240 Elemental Analyzer; Perkin-Elmer Corp., Norwalk, Conn.

[16] Karl Fisher analyses were performed on a Methohm Herisau-K.F. Automate E547 and Dosmat E535.

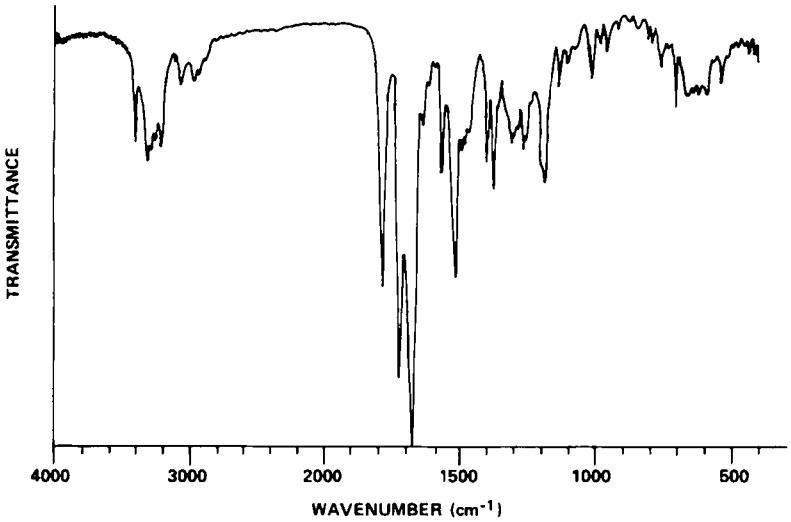


Figure 3—*IR spectrum of the unknown piperacillin impurity and synthetic piperacillin amide.*

7.72 ppm in the unknown piperacillin impurity. These differences in chemical shift, attributed to differences in concentration between the two samples, were verified in a NMR spiking experiment of synthetic piperacillin amide into the piperacillin impurity. Chemical shifts of amide protons undergo large variations when the hydrogen bonding interactions are modified by concentration, pH, temperature, and solvent changes.

The ^{13}C-NMR spectrum of the unknown piperacillin impurity is identical (within experimental error) to that of piperacillin; hence, piperacillin and the unknown impurity have identical carbon backbone structures. The ^{13}C-NMR chemical shifts and intensities of the synthetic piperacillin amide were identical to that of the unknown impurity.

The IR spectrum of the unknown piperacillin impurity and that of the synthetic piperacillin amide were identical (Fig. 3). The characteristic absorption band assigned to the carbonyl stretching mode of the β-lactam ring is at 1780 cm^{-1}.

The unknown piperacillin impurity is structurally very similar to piperacillin, *viz.*, the β-lactam group is intact (IR) and the major NMR absorptions of piperacillin and the piperacillin impurity are nearly identical, with the exception of two proton resonances at 7.72 and ~7.3 ppm. The MS data indicate that the unknown structure has a molecular weight of 516, one mass unit less than piperacillin, and based on this even mass has an elemental composition of an even number of nitrogen atoms. The unknown piperacillin impurity has been shown, based on the comparison of liquid chromatographic and MS, NMR, and IR spectrometric data, to be identical with *bona fide* piperacillin amide. The origin of the piperacillin amide is unknown.

Piperacillin amide was tested for biological activity in an *in vitro* spectrum assay against 27 bacteria. It lacked antimicrobial activity.

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