High-Pressure Liquid Chromatographic Quantitation of Azlocillin

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We describe a rapid, precise, and simple procedure for the quantitation of azlocillin in serum and in aqueous solutions by high-pressure, reverse-phase liquid chromatography. This method uses a single precipitation step, detection by dual-wavelength monitoring (220 and 254 nm), and quantitation by comparison with an internal standard. The 10- μ m C₁₈ μ Bondapak column was eluted with 45% (vol/vol) methanol-65% (vol/vol) phosphate buffer. The precision was 4.2 and 5.6% at 50 and 10 μ g/ml, respectively. The assay was linear up to concentrations of 100 μ g/ml, with recoveries of 90.4 to 110.5% from serum. The assay was sensitive to 0.4 μ g of azlocillin per ml. The short turnover time (14 min) and small serum sample size (20 μ l) make the assay ideal for therapeutic drug monitoring and clinical pharmacokinetic studies.

Azlocillin, a semisynthetic penicillin {6-[D-2-(2-oxoimidazolidine-1-carboxamido)-2-phenylacetamido]-penicillanic acid, sodium salt}, is a potent β -lactam antibiotic, particularly active against *Pseudomonas aeruginosa* (2, 3, 9, 12, 14).

Azlocillin has been quantitated in body fluids primarily by the agar disk diffusion assay. Test organisms include *P. aeruginosa* (13), *Bacillus subtilis* (5, 8), and *Micrococcus luteus* (1). The microbiological assay has inherently poor precision, has possible interference from other antibiotics present, and requires a minimum of 6 to 8 h of incubation, precluding dosage adjustment within a dosing interval.

High-pressure liquid chromatography (HPLC) is a rapid, reliable, and precise method for the quantitation of certain antibiotics. Three methods are described for azlocillin; two use a gradient solvent system (4, 10), and the other uses a reverse-phase system (6). None of the methods uses dual-wavelength monitoring, which ensures specificity of detection (7). In addition, none of the methods employs quantitation by reference to an internal standard, an approach which eliminates the errors introduced by variability of injection volume and nonuniform extraction (15).

We therefore developed a reverse-phase HPLC method which uses the common C₁₈ μ Bondapak column, dual-wavelength monitoring, and internal-standard quantitation. The method also employs single-step precipitation of serum, the only sample preparation necessary.

MATERIALS AND METHODS

An HPLC pump which could deliver a mobile phase at a constant flow rate of up to 4,500 lb/in² was used (Constametric I, Laboratory Data Control, Rivera Beach, Calif.). An injection system with a Waters Intelligent Sample Processor (model no. 710B, Waters Associates, Milford, Mass.) was programmed to inject 30 μ l per sample and was utilized with the above pump. A 10- μ m Waters μ Bondapak C₁₈ reverse-phase column (30 cm by 3.9 mm [inside diameter]; P/N 27324, Waters Associates) was employed. The mobile phase was monitored in series with a fixed-wavelength UV detector set at 254 nm (0.008 absorbance unit, full scale), and a variable-wavelength UV detector set at 220 nm (0.100 absorbance unit, full scale) models UV III and SpectroMonitor III, respectively). Both were purchased from Laboratory Data Control. A dual-pen recorder set at 0.5 cm/min (Omniscrite recorder, model no. B5217-5, Houston Instruments, Austin, Tex.) received signals from both detectors.

Azlocillin was kindly supplied by Elizabeth Griffith, Miles Pharmaceuticals, West Haven, Conn. A 1.0-mg/ml stock solution was prepared in distilled water and stored at -70°C.

Chloramphenicol was obtained from Sigma Chemical Co., St. Louis, Mo. Rifampin was obtained from Calbiochem, La Jolla, Calif. Cefazolin and cefamandole were obtained from Eli Lilly & Co., Indianapolis, Ind., and HR 756 was obtained from Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J. Salicylic acid, acetaminophen, ampicillin, and chloramphenicol succinate were obtained from this hospital's pharmacy.

The internal standard used for this assay, *p*-nitropropionanilide, was prepared by combining 10 mg of *p*-nitroaniline (Aldrich Chemical Co., Milwaukee, Wis.),

50 μ l of pyridine, and 100 μ l of propionic anhydride in a small vial with a Teflon-lined cap. The mixture was heated at 37°C under a stream of air. The residue was crystallized from an ethanol-water mixture. These crystals were dissolved in acetonitrile (Burdick-Jackson Laboratories, Muskegon, Mich.) at a concentration of 25 μ g/ml and stored at 5°C. Under these conditions, the internal standard was stable for 3 months.

The mobile phase consisted of 45 to 55% (vol/vol) methanol and phosphate buffer (67 mmol/liter), pH 3.5, at 22°C. Glass-distilled methanol (MCB Manufacturing Chemists, Cincinnati, Ohio) was mixed with the buffer, filtered with a 0.22- μ m filter (type GS, Millipore Corp., Bedford, Mass.), and degassed in vacuo.

The standards were prepared from the 1.0-mg/ml stock solution. The concentrations of the working standards were 100, 50, and 0 μ g/ml in distilled water. Quality control samples were prepared by diluting the stock solution in calf serum to yield concentrations of 10 and 50 μ g/ml. The working standards and quality control samples were stored at -70°C.

A volume (≥ 20 μ l) of the standards, controls, or serum samples was diluted with an equal volume of the internal standard. The mixture was vigorously mixed and centrifuged at ca. 10,000 $\times$ g (Eppendorf centrifuge model no. 5412, Brinkmann Instruments Inc., Westbury, N.Y.) for 2 min. Thirty microliters of the supernatant was injected into the column, and the

peak heights were measured. The ratio of the antibiotic peak height to the internal-standard peak height was determined. The azlocillin concentration was calculated by comparison of the peak height ratios with those of known standards by a least-squares regression analysis.

Linearity. A stock solution of azlocillin was diluted with distilled water to concentrations of 100, 75, 50, 25, 12.5, and 6.25 μ g/ml. Linear regression was used to calculate the slope and the correlation coefficient.

Sensitivity. The working standard was serially diluted with distilled water and assayed until the injected sample yielded a signal on the chromatogram at 220 nm, which was twice the height of background noise. The concentration of azlocillin in this diluted sample was defined as the sensitivity of the assay.

Precision. Ten samples of the high and low quality control samples were assayed on the same day. A sample of each control was then assayed daily for 7 days. Concentrations of high and low control were 50 and 10 μ g/ml, respectively. Within-run precision and run-to-run precision were determined from these data.

Stability. Samples of the high and low quality control specimens were stored at -70, -20, and 5°C for 1 month. Each sample was assayed at 0, 1, 2, and 4 weeks. Four samples were assayed and then reassayed 4 to 5 hours later at 22°C to determine within-run stability.

Recovery. The azlocillin stock solution was diluted

FIG. 1. Representative chromatograms. (A) Internal standard diluted with an aqueous sample (distilled water). (B) Aqueous standard containing azlocillin at 50 μ g/ml with the internal standard. (C) Human serum with azlocillin added at a final concentration of 50 μ g/ml with the internal standard. Absorbance at 220 nm ($A_{220\text{nm}}$) is shown at a scale of 0.1 absorbance unit, full scale. Retention times are 7.6 and 13.2 min for azlocillin and the internal standard, respectively. (D) Human serum without azlocillin added. The arrow indicates the azlocillin retention time.

FIG. 2. Stability of azlocillin when stored at three temperatures: 1 to 5°C (●), -20°C (◆), and -70°C (▲). Two concentrations are represented. Broken lines represent a 10-µg/ml concentration, and the solid lines depict a 50-µg/ml concentration.

in water and human serum to yield concentrations of 100, 50, and 10 µg/ml. The absolute peak heights of the dilutions in human serum were compared with the absolute peak heights of the antibiotic dissolved in water.

Specificity. A 1-mg/ml solution of ampicillin, salicylic acid, and acetaminophen was injected into the system and then coinjected with azlocillin. A 200-µg/ml solution of cefazolin, cefamandole, and HR 756 was prepared and injected and coinjected with azlocillin. Aqueous solutions (20 µg/ml) of chloramphenicol, chloramphenicol succinate, and rifampin were prepared, injected, and coinjected into the HPLC system. The peak heights at 220 nm were routinely used to determine concentrations. However, the ratio of the peak height at 220 nm to the peak height at 254 nm was monitored to ensure specificity. This ratio, 0.022 ± 0.008 , remained constant. A value outside this range indicated an interfering substance.

Bioassay. Serum samples from a healthy adult who received a dose of 7 g were assayed by the HPLC technique and an agar disk diffusion method (11), using *M. luteus* ATCC 9341. All samples and eight standards from 0 to 10 µg/ml were assayed in triplicate on medium 1 agar (Difco Laboratories, Detroit, Mich.), which was seeded with 10^6 organisms per ml of agar. The plates were then refrigerated at 1 to 5°C and used within 1 week. By using the HPLC values as the y axis

and the values from the microbiological method as the x axis, a linear regression was performed.

RESULTS

Representative chromatograms are depicted in Fig. 1 (see the legend for a description).

A standard curve was calculated using seven concentrations, including a zero point. The ratio of the antibiotic peak height to the internal-standard peak height was plotted against known azlocillin concentrations. The square of the correlation coefficient (r^2) was 0.9978 for azlocillin. The slope of the regression line was 1.0288, and the y intercept was 0.0432. Because of this excellent correlation, only three concentrations were used to define the standard curve on a routine basis.

With a 30-µl injection volume, the sensitivity of azlocillin detection (as defined) was 0.4 µg/ml.

Results of the precision test for azlocillin are as follows. The coefficient of variation was 4.2% ($n = 10$) for the high control and 5.6% ($n = 10$) for the low control when these samples were assayed on the same day. The coefficient of variation of the samples that were assayed on different days was 2.8% for the high control and 10.0% for the low control ($n = 7$). Standard deviations were 1.93, 0.68, 1.33, and 1.05, respectively.

Stability data for azlocillin are depicted in Fig. 2. Azlocillin in serum was stable for less than 1 week at -20 and 5°C but appeared to be stable for up to 1 month at -70°C. More than 50% of the drug decomposes in both the low and the

FIG. 3. Comparison of azlocillin concentrations when quantitated by HPLC technique and the agar disk diffusion method. Each circle represents one determination by both assays. The line shown is calculated by linear regression; its slope is 0.6278, and the y intercept is 5.096. The square of the correlation coefficient is 0.9578.

high quality control samples when stored at refrigerator temperatures (1 to 5°C) for 4 weeks. A 25% loss of assayable azlocillin when it is stored in aqueous solution at 20°C for 24 h has been previously reported (3). There was a detectable loss of azlocillin in serum or aqueous solution after 5 h at 22°C.

The range of recovery for concentrations between 20 and 200 µg/ml was 90.4 to 110.5%; the average was 101.1%. Serum containing no added azlocillin had an absorbance at 220-nm peaks of 2.0 mm or less 7.6 min. after injection (the time of azlocillin elution), which was the same as the background. The difference between the absolute peak heights of serum compared with the aqueous solutions of the antibiotic at the same concentration was insignificant ($P > 0.1$).

Ampicillin, rifampin, salicylic acid, cefazolin, HR 756, and acetaminophen were not detectable under these conditions; interference of azlocillin quantitation by these compounds was not observed. With cefamandole, two peaks were observed at retention times of 4.8 and 7.0 min. When coinjected, all three peaks eluted separately. Chloramphenicol had a retention time of 6.5 min, whereas chloramphenicol succinate had a retention time of 8.4 min. When these compounds were coinjected with azlocillin, all three eluted separately. The ratio of the peak heights at the two different wavelengths ensured correct identification of the azlocillin peak.

A comparison of the azlocillin concentration as determined by this method and the bioassay is shown in Fig. 3. The correlation coefficient was 0.9578, and a slope of 0.6278 was calculated. The results indicate a good correlation between the two assays, with, however, a consistent bias with one of the assays. Since this method is linear from 0 to 100 µg/ml (the bioassay is linear from 0.5 to 10 µg/ml) and demonstrates better precision and accuracy, we conclude that the bioassay overestimates azlocillin.

DISCUSSION

A reliable, rapid, and accurate assay of azlocillin is necessary for dosage adjustment during therapy. We describe an assay with good linearity, sensitivity, precision, recovery, and specificity. The method is simple and inexpensive when large numbers of samples are to be quantitated and HPLC equipment is available.

This method can be scaled down, using only 20 µl of a sample, if necessary. This allows blood sampling by fingertip puncture and the heel prick method.

This technique permits the adjustments of azlocillin dosage to maintain the serum concentration above the minimum inhibitory concentration and the minimum bactericidal concentration required for the infecting organism. The assay will also be of benefit in documenting the penetration of azlocillin into specialized body compartments. This is important when the infection is in an area where antibiotic penetration is variable (e.g., cerebrospinal fluid).

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