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High-Pressure Liquid Chromatographic Analysis of Aztreonam in Sera and Urine

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Aztreonam (SQ 26,776) is a new synthetic monocyclic β-lactam antibiotic which is specifically active against aerobic gram-negative bacteria. High-pressure liquid chromatographic (HPLC) systems were developed for the quantitative analysis of aztreonam in human, monkey, rat, mouse, and rabbit sera and urine. The HPLC conditions employed for these analyses were a μBondapak C_{18} column, a mobile phase made up of 0.005 M tetrabutylammonium hydrogen sulfate at pH 3.0 and acetonitrile or methanol, UV detection at 293 nm, and a flow rate of 2.0 ml/min. For human sera and urine, the mobile phase was 80% 0.005 M tetrabutylammonium hydrogen sulfate–0.005M $(NH_4)_2SO_4$ and 20% acetonitrile (vol/vol). For the range of sera and urine, HPLC analyses were shown to have excellent detector linearity of aztreonam over a concentration range of 1.0 mg/ml to 0.5 μg/ml. Correlation coefficients for plots of aztreonam peak area versus its concentration were ≥0.990. The detection limit of aztreonam was 1.0 μg/ml in sera and 5.0 μg/ml in urine. HPLC and microbiological assays of aztreonam in human sera and urine were in good agreement.

Aztreonam (SQ 26,776) (Fig. 1) is a totally synthetic monocyclic β-lactam antibiotic specifically active against aerobic gram-negative bacteria (6). It is a member of the monobactams, naturally occurring β-lactams produced by bacteria (7). This compound is currently undergoing clinical development, and human pharmacology data have been reported in detail (4, 5).

The assay of antibiotics in body fluids has traditionally been performed by microbiological assay, but recently high-pressure liquid chromatography (HPLC) has been extensively employed as an alternative procedure (8, 11). In comparison with the microbiological assay, HPLC analysis offers the advantages of increased speed of analysis and the ability to separate drug metabolites.

This report deals with HPLC systems for the quantitative analysis of aztreonam in the sera and urine of animals and humans which have been run in comparison with standard microbiological methods.

(A partial account of this work has been presented [F. G. Pilkiewicz, S. M. Fisher, B. J. Remsburg, and R. B. Sykes, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 882, 1981].)

MATERIALS AND METHODS

HPLC systems. HPLC separations were developed for the quantitative analysis of aztreonam in sera and urine from the following species: humans, cynomolgus monkeys, rats, mice, and rabbits. Two HPLC systems were used for these studies. One consisted of the following components: two M-6000A solvent delivery pumps, a model 660 solvent gradient programmer, a model U6K injector, (all from Waters Associates, Milford, Mass.), a model LC75 variable-wavelength UV detector and autocontroller (The Perkin-Elmer Corp., Norwalk, Conn.), and a model 3390A printer and plotter integrator (Hewlett-Packard, Avondale, Pa.). The other HPLC system was a Hewlett-Packard model 1084B HPLC which was equipped with a built-in variable-wavelength UV detector, autosampler, and printer and plotter integrator. The units yielded equivalent results and were used interchangeably. With both chromatographs the same column system was used: a Waters Associates μBondapak C_{18} column (inside diameter, 3.9 mm; length, 30.0 cm) and a Waters Associates guard column (inside diameter, 3.9 mm; length, 3.0 cm). The guard column was packed with Bondapak C_{18} on Corasil. For studies with both chromatographic systems, the following conditions were employed: a UV detector setting of 293 nm, a solvent flow rate of 2.0 ml/min, and a chart speed of 0.5 cm/min. Quantitation of aztreonam was performed by an external standard method of calculation, and peak area measurements were used.

Aztreonam. Aztreonam, prepared in our laboratories, was used as either the dipolar ion form shown in Fig. 1 or the disodium or dipotassium salt formed from the free diacid.

Chemicals. Acetonitrile and methanol were HPLC-grade solvents (Fisher Scientific Co., Pittsburgh, Pa.). Tetrabutylammonium hydrogen sulfate ($TBAHSO_4$) was purchased from the Aldrich Chemical Co., Mil-

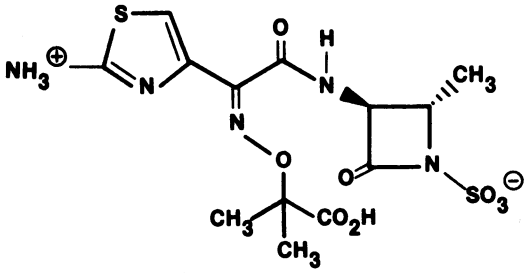

FIG. 1. Structure of aztreonam (SQ 26,776).

waukee, Wis., and ammonium sulfate, analytical reagent grade, was from Mallinckrodt, St. Louis, Mo. K_2HPO_4 was Fisher-certified American Chemical Society grade. Distilled and deionized water obtained from a Millipore Milli-Q System (Millipore Corp., Bedford, Mass.) was used for all studies.

Preparation of the HPLC mobile phase. A 0.005 M $TBAHSO_4$ solution adjusted to pH 3.0 with 1.0 M K_2HPO_4 was filtered through a 0.50-μm Fluoropore membrane filter (Millipore Corp.) and degassed before HPLC use. A 0.005 M $TBAHSO_4$ and 0.005 M $(NH_4)_2SO_4$ solution at pH 3.0 was prepared in a similar manner. Acetonitrile and methanol, the other liquid chromatography solvents, were also filtered and degassed.

Biological fluids. Urine and sera were obtained from the following sources: CD1 female mice and Holtzman female rats, Charles River Breeding Laboratories, Inc., Wilmington, Mass.; New Zealand white female rabbits, Summit View Farms, Belvidere, N.J.; and male and female cynomolgus monkeys, Hazleton/Primelabs, Inc., Farmingdale, N.J. Human urine was donated by healthy male volunteers and pooled, whereas sera were obtained from both healthy male volunteers and GIBCO Laboratories, Grand Island, N.Y., and pooled.

HPLC studies of aztreonam in sera. To determine a concentration range with a suitable UV detector linearity and to test the efficiency of a serum sample preparation technique, standards of aztreonam dissolved in the HPLC mobile phase were prepared at concentrations of 1,000, 500, 200, 100, 50, 20, 10, 5, 1, and 0.5 μg/ml. Standards were analyzed by HPLC, and a standard curve of aztreonam peak area versus concentration was constructed. For each of the different sera, aztreonam standards in sera were prepared over the same concentration range as the standards dissolved in the HPLC mobile phase. Before HPLC analysis, serum standards were diluted with an equal volume of acetonitrile and centrifuged for 2 min at an ambient temperature and 15,000 × *g* in an Eppendorf model 3200 centrifuge. Supernatants were removed and subjected to HPLC analysis under the same conditions as those used for the standards dissolved in the mobile phase. Peak areas of aztreonam obtained from serum standards were plotted on the earlier constructed standard curve of aztreonam standards dissolved in the HPLC mobile phase. As the sample preparation technique was efficient for all sera, standards were made up in the respective sera over the 1,000- to 0.5-μg/ml concentration range, and both standards and samples were prepared for analysis by using the described preparation technique. The HPLC-analyzed serum standards were used in constructing calibration curves for determining the amount of aztreonam present in serum samples.

HPLC studies of aztreonam in urine. Aztreonam standards were prepared in urine for each of the different species studied over the same concentration range as that used in the preparation of the calibration curve of standards dissolved in the HPLC mobile phase (1,000 to 0.5 μg/ml). Urine samples were diluted 10-fold with 0.005 M $TBAHSO_4$ (pH 3.0) and analyzed by HPLC under the same conditions as the standards dissolved in the mobile phase. When peak areas of urine standards were plotted on the calibration curve of the HPLC mobile phase dissolved standards, the results showed that the urine sample preparation technique was efficient.

Based on these findings, all of the urine studies were done by preparing standards of aztreonam in the respective urine, preparing the standards and urine samples for HPLC analysis in the manner described above, and using the HPLC-analyzed urine standards in constructing a calibration curve for determining the concentration of aztreonam in the urine samples.

Intravenous dose study of aztreonam in humans. Forty-eight healthy male subjects (six groups with eight subjects per group) took part in the study. The study was carried out as described previously (4).

Upon receipt of the sera and urine, samples were prepared for HPLC analysis in the manner described earlier and stored at −20°C if not immediately analyzed.

Microbiological assay of aztreonam. Aztreonam concentrations in sera and urine were determined by a microbiological agar diffusion method with *Escherichia coli* SC12155 as the test organism. Serum samples prepared in pooled human sera were initially diluted 1:20 in 0.1 M phosphate buffer (pH 6.0). Subsequent dilutions of test levels were made in a diluent of 5% serum and 95% buffer. The quantitation limit of the method was 0.40 mg/liter. Serum samples containing less than this concentration were assayed in a more sensitive system capable of quantitating aztreonam concentrations as low as 0.06 mg/liter. This latter system involved the extraction of serum standards and samples with methanol, centrifugation, and dilution of the supernatants with 0.1 M phosphate buffer (pH 6.0). Standards were prepared in phosphate buffer (pH 6.0) for the assay of the urine samples. The quantitation limit of the urine assay method was 0.1 mg/liter (4).

RESULTS

HPLC conditions presented in Table 1 were found to be optimal for the analysis of aztreonam in the various biological fluids. The HPLC systems described in Table 1 for rat, mouse, and monkey sera were also used to confirm the results of serum pharmacokinetic studies on these animals obtained with standard microbiological methods (1). The HPLC system in Table 1 for human sera and urine was also used to determine the amount of aztreonam present in the sera and urine of human volun-

TABLE 1. HPLC mobile phase for the analysis of aztreonam in serum and urine samples[a]

Biological fluid	Mobile phase (vol/vol)
Mouse serum	80% 0.005 M $TBAHSO_4$ and 20% CH_3CN
Mouse urine	80% 0.005 M $TBAHSO_4$ and 20% CH_3CN
Rat serum	65% 0.005 M $TBAHSO_4$ and 35% CH_3OH
Rat urine	82.5% 0.005 M $TBAHSO_4$ and 17.5% CH_3CN
Rabbit serum	75% 0.005 M $TBAHSO_4$ and 25% CH_3CN
Rabbit urine	82.5% 0.005 M $TBAHSO_4$ and 17.5% CH_3CN
Cynomolgus monkey serum	65% 0.005 M $TBAHSO_4$ and 35% CH_3OH
Cynomolgus monkey urine	80% 0.005 M $TBAHSO_4$ and 20% CH_3CN
Human serum	80% 0.005 M $TBAHSO_4$–0.005 M $(NH_4)_2SO_4$ and 20% CH_3CN
Human urine	80% 0.005 M $TBAHSO_4$–0.005 M $(NH_4)_2SO_4$ and 20% CH_3CN

[a] For the HPLC analyses of the serum and urine samples, the following chromatographic conditions were used: a Waters Associates μBondapak C_{18} column (inside diameter, 3.9 mm; length, 30.0 cm) and a Waters Associates guard column (inside diameter, 3.9 mm; length, 3.0 cm) packed with C_{18} Bondapak on Corasil, a UV detector wavelength of 293 nm, and a flow rate of 2.0 ml/min. $TBAHSO_4$ and $TBAHSO_4$–$(NH_4)_2SO_4$ had a pH of 3.0.

teers from a single-intravenous-dose study of the drug (4, 5).

Chromatograms of a blank human serum sample and a human serum sample containing aztreonam are shown in Fig. 2. There were no liquid chromatography peaks arising from the blank serum or from the serum of the dosed volunteer which interfered with the assay. A similar comparison of chromatograms shows that the human urine HPLC assay of aztreonam was also free from interfering peaks (Fig. 3). By using the HPLC conditions given in Table 1, it was possible to analyze urine and sera from all of the listed animal species for aztreonam without encountering interfering UV-absorbing liquid chromatography peaks.

The detector linearity for HPLC assays of aztreonam in the different sera and urine over a wide concentration range was excellent. The use of linear regression analysis for the calibration curves of aztreonam concentration versus its peak area for either the sample-prepared sera or urine over an aztreonam concentration range of 1.0 mg/ml to 0.5 μg/ml gave correlation coefficients of ≥0.990. A calibration curve of aztreonam standards dissolved in the HPLC mobile phase over a concentration range of 1.0 mg/ml to 0.05 μg/ml gave a correlation coefficient of 1.00 via linear regression analysis. The HPLC system used for these standards was the one listed in Table 1 for human sera and urine.

The sample preparation techniques for all sera involving a twofold dilution with acetonitrile before HPLC analysis yielded satisfactory results. By employing the calibration curve of aztreonam standards dissolved in the mobile phase to determine the efficiency of the sample preparation technique, it was found that the amount of aztreonam present in the processed sera over a concentration range of 1.0 mg/ml to 0.5 μg/ml was ≥95 and ≤103% of the actual amount. The sample preparation technique employed for urine was a 10-fold dilution with pH 3.0-adjusted 0.005 M $TBAHSO_4$. The use of the calibration curve of aztreonam standards dis-

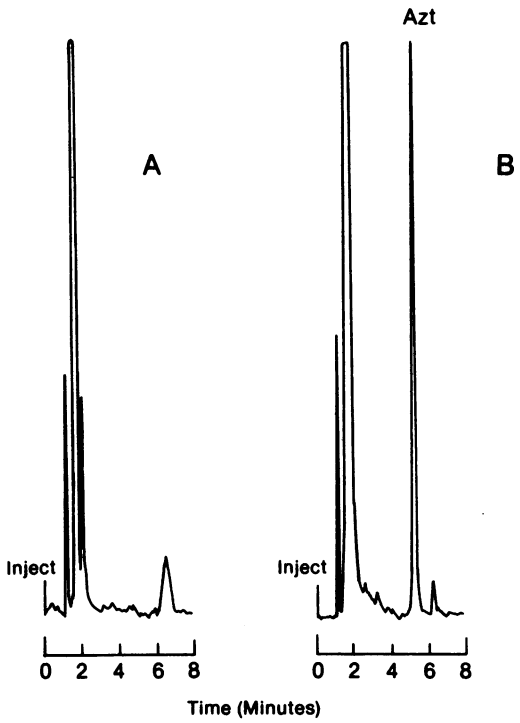

FIG. 2. HPLC analysis of aztreonam (Azt) in human sera. (A) Blank human serum sample; (B) human serum sample containing aztreonam at a concentration of 4.23 μg/ml. HPLC conditions were as follows: instrument, Hewlett-Packard 1084B HPLC; column, Waters Associates μBondapak C_{18} column (inside diameter, 3.9 mm; length, 30.0 cm); mobile phase, 80% 0.005 M $TBAHSO_4$–0.005 M $(NH_4)_2SO_4$ at pH 3.0 and 20% CH_3CN (vol/vol); flow rate, 2.0 ml/min; detector, Hewlett-Packard variable-wavelength UV detector; λ, 293 nm; absorbancy, 0.0008; injection volume, 50 μl.

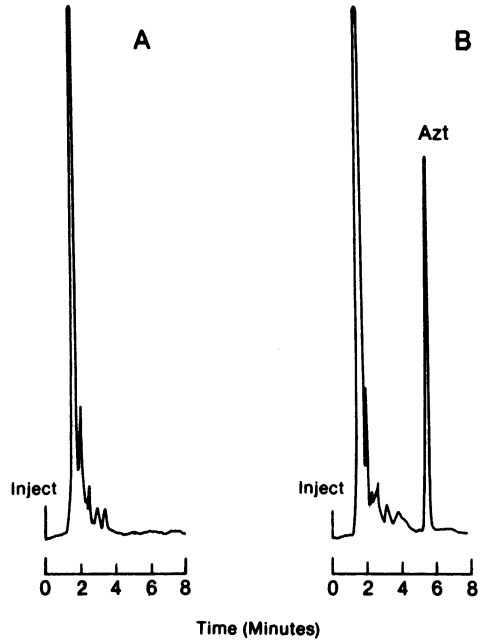

FIG. 3. HPLC analysis of aztreonam (Azt) in human urine. (A) Blank human urine sample; (B) human urine sample containing aztreonam at a concentration of 14.93 μg/ml. HPLC conditions were the same as listed in the legend to Fig. 2 except that in (A) the absorbancy was 0.0008 and in (B) the absorbancy was 0.0032.

solved in the mobile phase in determining the efficiency of the urine sample preparation technique over the same 1.0-mg/ml to 0.5-μg/ml concentration range of aztreonam resulted in recoveries of ≥96 and ≤102%.

The HPLC detection limit for the quantitation of aztreonam was 1.0 μg/ml in sera and 5.0 μg/ml in urine. However, the method can be used to qualitatively detect aztreonam at concentrations of ≤0.5 and ≥0.025 μg/ml. In Fig. 4 an HPLC chromatogram of aztreonam at a concentration of 0.05 μg/ml is shown. The time of analysis for either serum or urine was less than 10.0 min per sample.

There was good agreement between the HPLC and microbiological assays for both serum and urine samples collected from the single-intravenous-dose study in humans. A plot of the median values of the HPLC versus the microbiological assay results for the concentration of aztreonam in sera for a 0.5-g intravenous dose is shown in Fig. 5. The correlation coefficient obtained via linear regression analysis was 0.997. Figure 6 shows a plot of the median aztreonam values in urine of both assays for the 0.5-g intravenous dose. The correlation coefficient by linear regression analysis was 0.998.

DISCUSSION

In comparing the HPLC assay of aztreonam with a standard microbiological assay, the HPLC assay offers advantages in speed, the need for less sample volume, and the ability to separate chemical entities. HPLC analysis time was less than 10 min per sample and could be performed with as little as 10 μl of urine or 50 μl of serum. Moreover, for the biological fluids studied, the HPLC assay was free from interfering compounds.

Microparticulate reverse-phase columns are widely used for the HPLC analysis of β-lactam antibiotics (2, 3, 9, 10). Ion pair chromatography is employed with reverse-phase columns when dealing with a compound which is in an ionic form in the mobile phase and has to be ion paired to be retained on the column. Such methodology has recently been employed in analyzing fermentation broths containing cephalosporin C and its metabolites (10). The SO_3H group of aztreonam makes the compound a strong acid ($pK_a < 2.0$); as such, it must be ion paired for retention on a reverse-phase μBondapak C_{18} column. This was achieved with $TBAHSO_4$ which, when used with acetonitrile, gave a good retention time and a symmetrical peak shape to

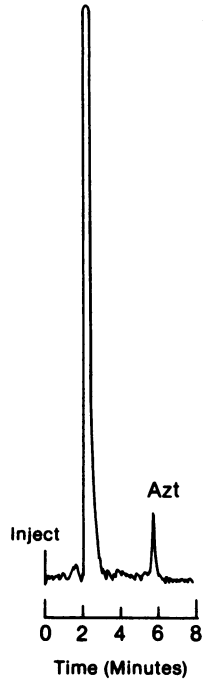

FIG. 4. HPLC analysis of aztreonam (Azt) at low concentrations. The concentration of aztreonam was 0.05 μg/ml. HPLC conditions were the same as listed in the legend to Fig. 2 except that absorbancy was 0.0004 and the injection volume was 200 μl.

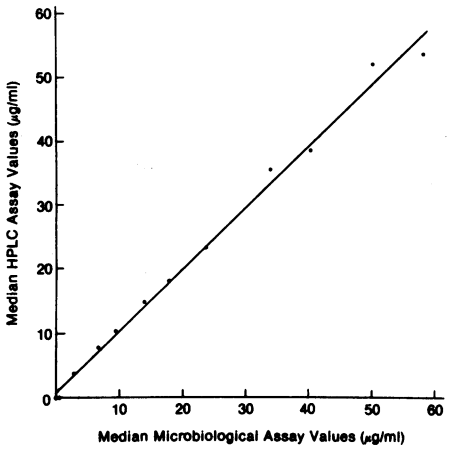

FIG. 5. Plot of the median concentrations of aztreonam in human sera for a 0.5-g-intravenous-dose study determined by HPLC versus the median values determined by microbiological assay. The correlation coefficient was 0.997.

aztreonam. Use of an equimolar mixture of $TBAHSO_4$ and $(NH_4)_2SO_4$ with the same amount of acetonitrile as employed with $TBAHSO_4$ alone lowered the retention time of aztreonam and sharpened its HPLC peak shape. This is because $(NH_4)_2SO_4$ forms an ion pair with aztreonam that is less organic in nature than a $TBAHSO_4$-aztreonam ion pair, and both ammonium ions contribute to the ion pair seen by the column. The use of $TBAHSO_4$ and $(NH_4)_2SO_4$ for analysis of biological fluids of human origin allowed aztreonam to be separated from biological fluid components which were atypical to most of the serum and urine samples. A feature of ion pair chromatography is that the dipolar ion of aztreonam, as well as its disodium and dipotassium salts, forms the same chemical species; therefore, the HPLC retention time for all three forms of aztreonam was the same.

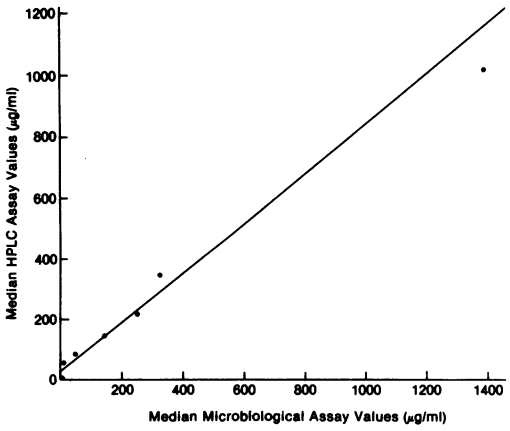

FIG. 6. Plot of the median concentrations of aztreonam in human urine for a 0.5-g-intravenous-dose study determined by HPLC versus the median values determined by microbiological assay. The correlation coefficient was 0.998.

The HPLC assays showed excellent detector linearity and accuracy over a 1,000-fold concentration range of aztreonam (from 1.0 mg/ml to 0.5 μg/ml). Below 0.5 μg/ml, due to detector noise, accuracy decreased.

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