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High-Pressure Liquid Chromatographic Assay of Netilmicin in Plasma

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A high-pressure liquid chromatographic method for the quantitative determination of netilmicin in plasma was developed. The procedures involve acetonitrile protein precipitation, methylene chloride extraction, and dansylation to form the fluorescent dansyl derivative of netilmicin, which is extracted into ethyl acetate and chromatographed on a reverse-phase column with aqueous acetonitrile as the mobile phase. A good linear relationship between peak height measurements and netilmicin concentrations was found. This method is sensitive and reproducible; a netilmicin concentration as low as 0.5 μg/ml can be measured with only 0.1 ml of plasma sample. The results of assays of plasma or serum samples by this high-pressure liquid chromatographic method correlate well with those obtained by microbiological assays.

Netilmicin is a new semisynthetic derivative of sisomicin. Its antimicrobial spectrum and activity are similar to those of other structurally related aminoglycosidic antibiotics such as gentamicin, sisomicin, and tobramycin (5, 6, 9). Although compared with gentamicin, netilmicin demonstrated lower nephrotoxicity in animals (3, 4) and less variation in peak levels after intramuscular administration in humans (7), it has potential for plasma level variations and oto- and nephrotoxicity. We wish to report a high-pressure liquid chromatographic method for determining plasma or body fluid levels of netilmicin. Such an assay could be useful in dosage regimen adjustments, in maintaining therapeutic levels in plasma, in averting untoward reactions, and in obtaining useful pharmacokinetic data. Similar benefits have been derived from the assays of gentamicin in plasma (2, 8).

MATERIALS AND METHODS

Netilmicin was obtained from Schering Corp., Bloomfield, N.J. 5-Dimethylamino-1-naphthalene sulfonyl chloride (dansyl chloride) was purchased from Sigma Chemical Co., St. Louis, Mo. Glass-distilled acetonitrile, methylene chloride, and ethyl acetate were from Burdick and Jackson Laboratory, Muskegon, Mich. Sodium carbonate, sodium bicarbonate, and phosphoric acid were reagent grade from Fisher Scientific Co., Fair Lawn, N.J.

Sample preparation. Portions (0.1 ml) of plasma samples were diluted with 0.9 ml of phosphate buffer (0.1 M, alkalinized with sodium hydroxide to pH 11), placed in culture tubes (13 by 100 mm) containing 2.5 ml of acetonitrile, blended in a Vortex mixer, and centrifuged. The supernatant solutions were poured into new tubes, blended in a Vortex mixer with 2 ml of methylene chloride, and centrifuged at 2,000 rpm for about 1 min. Portions (0.5 ml) of the separated aqueous (upper) layer were transferred to new, screw-cap culture tubes containing 4 mg of dansyl chloride in 0.3 ml of acetonitrile. These tubes were closed and incubated in a water bath at 75°C for 5 min. The reaction mixtures were cooled in ice-water, and 0.5 ml of ethyl acetate and 6 ml of carbonate buffer (containing 0.5 M sodium carbonate and bicarbonate, pH 9.5) were added. After Vortex mixing and centrifugation, 1- to 5-μl portions of the organic (upper) layer were chromatographed.

Blank plasma samples supplemented with different concentrations of netilmicin (0.5 to 20 μg/ml) were prepared in the same way for chromatographic analysis. The results for these samples were used for constructing calibration curves. Plasma blanks and plasma samples containing kanamycin, chloramphenicol, sulfisoxazole, salicylates, ampicillin, acetaminophen, and gentamicin were also analyzed to investigate possible interferences.

Chromatographic conditions. A chromatographic system consisting of a liquid chromatograph (model 601, Perkin-Elmer Corp., Norwalk, Conn.) equipped with a sample injector (Glenco Scientific Inc., Houston, Tex.), a reverse-phase column (μ-Bondapak C_{18}, Waters Associates, Milford, Mass.), and a liquid chromatographic fluorometer (FS 970, Schoeffel Instrument Corp., Westwood, N.J.) was used in this investigation. The mobile phase, a mixture of acetonitrile and water (95:5, vol/vol), was pumped through the system at a rate of 1.0 ml/min. All separations were carried out at ambient temperature. For detection, the fluorescence excitation wavelength was set at 220 nm, and an interference filter (KV470) was used to select the emission

wavelength. The chromatograms were recorded on a 10-mV potentiometric recorder. Peak height measurements were used for quantitative determinations.

RESULTS

Typical chromatograms of netilmicin from a patient plasma sample and of plasma blanks with and without antibiotic supplementation are shown in Fig. 1. Netilmicin eluted as a single peak, with a retention time of 5.85 min under our chromatographic conditions. The plasma from healthy subjects and from patients on therapy with theophylline, procainamide, propranolol, and other drugs showed no interference for the assay. For plasma samples supplemented with various drugs, only gentamicin interfered with the assay. Under the experimental conditions, gentamicin eluted as two peaks, of which the peak with a longer retention time (5.9 min) eluted close to that of netilmicin.

Figure 2 shows a calibration curve of netilmicin in plasma. A good linear relationship was observed between peak height measurements and the drug concentrations in the range studied, even though no internal standard was used in the assay. The peak heights were fairly reproducible: for example, five replicate assays of samples containing 10 and 2 μg of drug per ml of plasma gave average peak heights of 5.64 and 1.12 cm, respectively, with coefficients of variation of 2.02 and 1.49%, respectively. However, since fluorescence intensity is subject to quenching and temperature fluctuations, it is advisable to analyze two or more plasma samples containing known amounts of netilmicin concurrently with samples from patients. These samples with known concentrations of drug serve as calibration standards and as a check of performance of the instrumental system. A plasma netilmicin concentration as low as 0.5 μg/ml can be measured by this assay method.

Some plasma and serum samples from patients on this antibiotic were analyzed by this procedure. A plasma netilmicin concentration profile of a patient after a 2.0-mg/kg intramuscular injection is shown in Fig. 3. The concentration reached peak level of 9.4 μg/ml at about 1.5 h after injection. From the results of up to 8 h of sampling, the apparent biological half-life appears to be about 2 h.

For comparison, some plasma samples were assayed both by this high-pressure liquid chromatographic and by microbiological methods. The latter were performed by the agar well diffusion technique, using Mueller-Hinton agar (Difco) seeded with *Bacillus subtilis* spores (7). Figure 4 depicts the correlation between the results obtained by these two methods. The

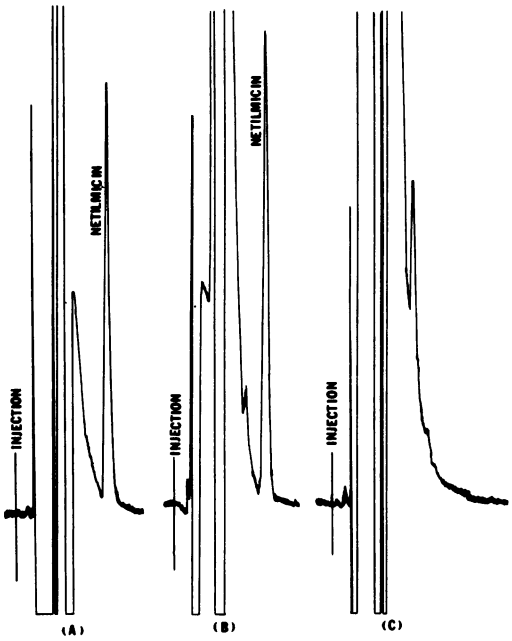

FIG. 1. *Chromatograms of netilmicin (A) from a patient plasma sampled 2 h post-intramuscular injection of 2 mg of netilmicin per kg and (B) from a plasma blank supplemented with 10 μg of netilmicin per ml. (C) Chromatogram of a plasma blank. Netilmicin retention time: 5.85 min.*

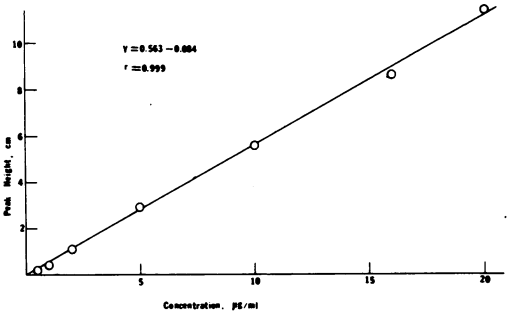

FIG. 2. *Calibration curve of netilmicin in plasma.*

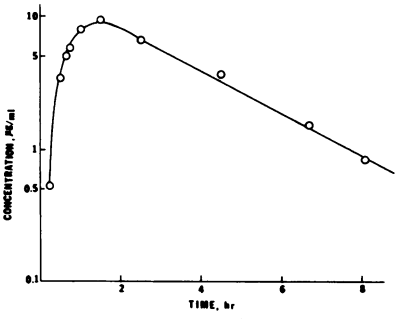

FIG. 3. *Concentration profile of netilmicin in plasma of a patient after a 2-mg/kg intramuscular netilmicin injection.*

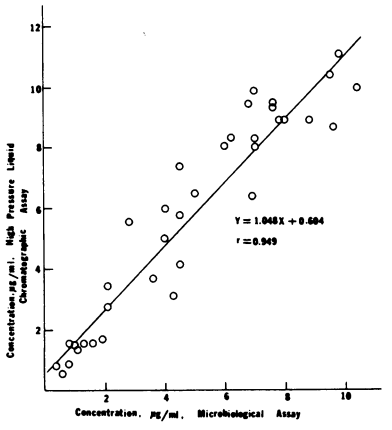

FIG. 4. *Correlation of the high-pressure liquid chromatographic assay of netilmicin in plasma with the microbiological assay.*

correlation from linear regression analysis gave an equation of $Y = 1.048X + 0.602$, with a correlation coefficient of 0.949 that was significant at $P < 0.00005$. Therefore, statistically there were no significant differences in the assay of plasma netilmicin by these two methods.

DISCUSSION

Netilmicin in plasma or body fluids can be analyzed by microbiological assay (7). A radioimmunoassay for netilmicin has also been reported (10). Although the former assay is simple and inexpensive and the latter is quite sensitive, they have some disadvantages in common with assays using biological agents or substances derived from biological sources (5a).

High-pressure liquid chromatography is well suited for analyzing many different types of compounds, both polar and nonpolar. However, when this method is applied to the analysis of aminoglycosidic antibiotics, the lack of fluorophore and chromophore presents special difficulties in detecting in the concentration ranges clinically encountered. The use of chemical reactions to form fluorescent or ultraviolet light-absorbing products can overcome these difficulties. Induction of fluorescence through precolumn dansylation of gentamicin, a method similar to the procedures described here, has been developed in this laboratory for assay of gentamicin in plasma (5a). A postcolumn fluorescence induction using *o*-phthalaldehyde has also been reported recently for the assay of gentamicin in serum (1).

Like that of gentamicin dansylation (5a), the product of the netilmicin dansylation reaction appears to be fairly nonpolar, since it eluted late from the column under the same chromatographic conditions.

The high-pressure liquid chromatographic assay of netilmicin described here is sensitive and accurate. Each assay requires only 0.1 ml of plasma sample, and the result can be obtained in less than 20 min. This method can be used for pharmacokinetic studies and for monitoring this antibiotic in plasma.

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