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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF VANCOMYCIN IN PLASMA, BONE, ATRIAL APPENDAGE TISSUE AND PERICARDIAL FLUID

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SUMMARY

Solid-phase extraction coupled with reversed-phase high-performance liquid chromatography and UV detection was employed for the analysis of the antibiotic vancomycin in patient plasma, bone, atrial appendage, and pericardial fluid. Vancomycin was quantitated in samples from patients undergoing cardiac surgery. Calibrations were linear in the range 3–100 μg/ml vancomycin; the lower limit of detection was approximately 3 μg/ml in fluids with an absolute limit of detection in bone samples of 0.75 μg per injection.

INTRODUCTION

Vancomycin is an amphoteric glycopeptide antibiotic, first isolated in 1956, that is active against a broad range of gram-positive bacteria as well as some gram-negative cocci. Because of toxicity, vancomycin was used primarily for alternative therapy before the recent emergence of methicillin-resistant and penicillin-resistant organisms [1,2]. For patients allergic to both the penicillins and cephalosporins, vancomycin is often the only effective treatment [1,2]. In addition to insuring that the patient is receiving a therapeutic dose, monitoring blood levels of vancomycin can help avoid ototoxicity and nephrotoxicity.

Several different analytical methods have been used to assay vancomycin in

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blood, including bioassay, high-performance liquid chromatography (HPLC), radioimmunoassay, fluorescence polarization immunoassay (FPIA), and fluorescence immunoassay. The precision and accuracy of these methods have been compared [3]; for clinical laboratories FPIA is the recommended methodology, because it is rapid and automated, although expensive. Even though HPLC takes longer than FPIA, HPLC is as precise and less expensive, once the initial investment in the instrumentation has been made.

A recent computer-assisted search using Medline found four different HPLC methods for quantitating vancomycin in blood in the literature: sample preparation by ion exchange followed by a reversed-phase (C_{18}) separation [4] and a modification of this method [5], sample preparation by liquid–liquid extraction and a reversed-phase (C_8) separation [6], liquid–liquid sample extraction and a reversed-phase (C_{18}) separation using a paired-ion mobile phase modifier [7], and finally sample pretreatment by protein precipitation with separation using a cyano (CN) HPLC column [8].

In this paper, we report an HPLC method for vancomycin employing a Bond Elut (C_8) solid-phase extraction of the plasma sample and a reversed-phase (C_8) separation. The method was applied to patient samples of plasma, bone, tissue, and pericardial fluid from a study involving cardiac surgery patients.

EXPERIMENTAL

Chemicals and reagents

Vancomycin hydrochloride and cefazolin sodium were supplied by Eli Lilly (Indianapolis, IN, U.S.A.) and Smith Kline (Philadelphia, PA, U.S.A.), respectively. All drug stock solutions in water were kept refrigerated at 2–8°C and found to be stable for at least two weeks with no change in concentration detectable by HPLC. Reagent-grade potassium dihydrogen phosphate and sodium hydroxide were obtained from Fisher Scientific (Pittsburg, PA, U.S.A.); HPLC-grade acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). All water was distilled. The HPLC solvent was filtered prior to use through a 0.45-μm FHUP filter from Millipore (Bedford, MA, U.S.A.). Sample clean-up columns employed were C_8 Bond Elut columns from Analytichem (Harbor City, CA, U.S.A.). The internal standard employed in all separations consisted of 250 μg/ml cefazolin in water prepared by serial dilution from a master stock solution (1 mg/ml) in water.

Instrumentation

The HPLC system consisted of a Model 6000 high-pressure pump, a Model 710B WISP autoinjector, a Model 440 UV absorbance detector, and a Model 840 data and chromatography control system (Waters Assoc., Milford, MA, U.S.A.). All centrifugation was carried out in a clinical centrifuge (Model CL, IEC, Needham Heights, MA, U.S.A.). Tissue homogenization was performed with an Ultra-Turex (Tekmar, Cincinatti, OH, U.S.A.), operated in series with a rheostat. Bone samples were blended in a Waring 7-speed blender (Model 13DL44, Waring, New Hartford, CT, U.S.A.). Lyophilization was carried out using equipment from Vir-

tis Research Equipment (Gardiner, NY, U.S.A.) connected to a dual-seal vacuum pump (Model 1402, Welch Scientific, Skokie, IL, U.S.A.). A Cary 210 spectrophotometer (Varian Assoc., Palo Alto, CA, U.S.A.) was employed for the UV scan of vancomycin and cefazolin.

All separations were performed at ambient temperatures (22–24°C) using a Supelcosil C_8 reversed-phase column (250 mm × 4.6 mm, 5 μm particle size) preceded by a C_8 guard column (50 mm × 4.6 mm, 40 μm particle size), both from Supelco (Bellefonte, PA, U.S.A.). The mobile phase consisted of 0.05 *M* potassium dihydrogen phosphate–acetonitrile (90:10, v/v), adjusted to pH 5.0 with 4 *M* sodium hydroxide. The column was operated isocratically at 1.5 ml/min with a pressure drop of 137.8 bar. Injection volumes were 150 μl for prepared plasma, pericardial fluid and atrial appendage samples, and 200 μl for bone extract samples. The detector was set at 254 nm, 0.05 a.u.f.s. Chromatographic analysis time was under 13 min.

Sample collection

The subjects of this study were twelve patients undergoing coronary artery bypass surgery where vancomycin was administered as the antibiotic for prevention of postoperative infection. A maximum of eight blood samples was obtained during the course of surgery for each patient. Blood samples were collected in heparinized tubes, centrifuged, and the plasma was stored at −70°C before assay. Single samples of bone, atrial appendage, and pericardial fluid were collected from each patient and stored in the same manner as the plasma.

Preparation of plasma samples

The C_8 Bond Elut sample clean-up columns were prepared just prior to use by washing with 1 ml of methanol and then 1 ml of water. To 0.5 ml of plasma, 15 μl of the internal standard cefazolin solution (250 μg/ml) were added. After vortexing, the sample was applied to a Bond Elut column. After centrifugation for about 30 s, the eluate was discarded and two successive 200-μl washings with methanol–water (50:50) were collected in a 15-ml conical tube. These washings were vortexed and left standing in ice for 3 min, after which the tube was vortexed and centrifuged again for 2 min. The supernatant was aspirated into a vial for injection of 150 μl into the HPLC system. The injection was performed within 1 h of completion of sample preparation. Bond Elut columns were cleaned after use by washing with 1 ml methanol followed by 1 ml of water and each Bond Elut column was discarded after a maximum of three uses.

Preparation of bone samples

Using shears, each bone sample was cut into 1-cm^2 pieces and kept frozen in dry ice. Several pieces of dry ice were ground in a blender; bone samples were then added and pulverized in the blender with the dry ice. The mixture was transferred to a beaker and the dry ice allowed to evaporate, after which the weight of the pulverized bone sample was recorded. Distilled water (10 ml) was added and the mixture sonicated in an ice bath for 20 min. The resulting sample mixture was centrifuged and the aqueous top layer removed. Care was taken to avoid removing

any fat layer which might have been floating. This aqueous layer was then processed as described in the next paragraph to analyze for loosely bound water-extractable drug amounts.

To the aqueous extract, 25 μl of solution containing the internal standard (cefazolin, 62.5 μg/ml) were added. This is less internal standard than that added to plasma, because lower amounts of vancomycin were expected. This mixture was lyophilized to reduce the volume of solution and reconstituted in 1 ml of water. The sample was processed through a Bond Elut column; 200 μl of this final solution were injected into the HPLC system.

Preparation of pericardial fluid samples

Pericardial fluid samples were prepared as described previously for the plasma samples.

Preparation of atrial appendage samples

Frozen tissue samples were cut into small pieces and weighed to the nearest 0.1 mg. Next, samples were homogenized at low speed for 2 min in 1 ml cold, deionized water while keeping the sample tube in ice. The sample was then centrifuged for 3 min and 0.5 ml of the supernatant were removed for analysis. The remainder of the tissue preparation was identical to that described previously for the plasma samples.

Calibration standards for plasma samples

Working standard stocks (31.35–1000 μg/ml) of vancomycin were prepared by serial dilution in water from a 10 mg/ml master stock solution. The standard calibration points (3.13–100 μg/ml) were prepared by adding 50 μl of the working standard to 450 μl of drug-free plasma and 15 μl of cefazolin (250 μg/ml). With each set of patient samples processed, two standard calibration points were freshly prepared. A master standard calibration was compiled from all these points by plotting the ratio of peak area of vancomycin to the peak area of cefazolin against concentration of vancomycin in plasma. A straight line fitted to these data was used to calculate the vancomycin concentration in the patient samples.

Calibration standards for bone samples

Working standard stocks (31.35–500 μg/ml) of vancomycin were prepared by serial dilution in water from a 10 mg/ml master stock solution. The standard calibration points (3.13–50 μg/ml) were prepared by adding 50 μl of the working standard to 450 μl of methanol–water (50:50), and 25 μl of cefazolin (62.5 μg/ml). With each set of patient bone samples processed, two standard calibration points were freshly prepared. A master standard calibration was compiled from all these points by plotting the ratio of peak area of vancomycin to the peak area of cefazolin against concentration of vancomycin. A straight line fitted to these data was used, along with the weight of bone sample, to calculate the vancomycin concentration in the patient bone samples.

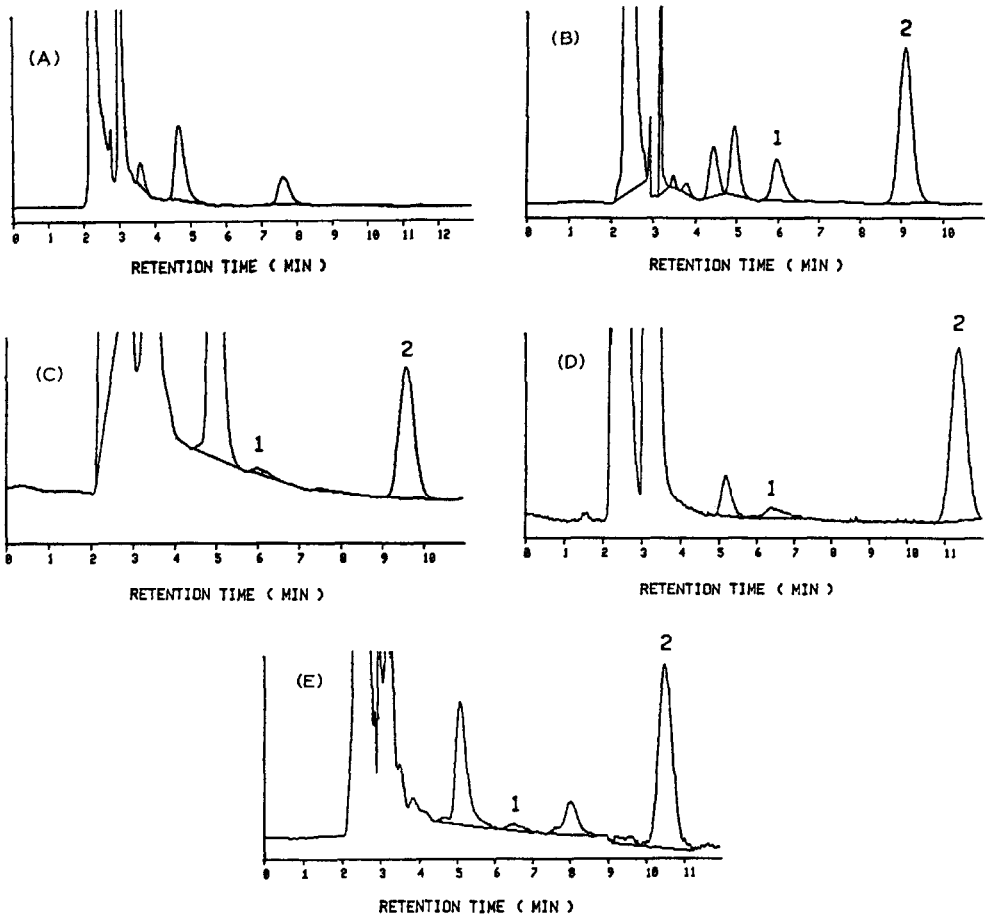

Fig. 1. Representative HPLC profiles of extracted patient samples. (A) Drug-free plasma; (B) plasma containing 15.3 μg/ml vancomycin; (C) xyphoid bone containing 2.36 μg/g vancomycin; (D) atrial appendage tissue containing 38.3 μg/g vancomycin; (E) pericardial fluid containing 5.53 μg/ml vancomycin. Peaks: 1 = vancomycin; 2 = cefazolin, internal standard.

Calibration standards for pericardial fluid and atrial appendage samples

Preparation of calibration curves for the pericardial fluid and atrial appendage samples was similar to that employed for bone samples, except that the range of vancomycin concentration was 3.13–25 μg/ml and the amount of internal standard cefazolin (250 μg/ml) added was 15 μl.

RESULTS

The chromatogram of a drug-free plasma sample is shown in Fig. 1A. Representative chromatograms from patient samples of plasma, bone, atrial appendage, and pericardial fluid are presented in Fig. 1B–E. Previous HPLC work [5,8] employed ristocetin as an internal standard. Ristocetin elutes before vancomycin under the chromatographic conditions used here; we did not find ristocetin to be a satisfactory internal standard because of interference from endogenous peaks

in the patient samples. Cefazolin was selected as an internal standard, because it elutes much later in the chromatogram and is only rarely interfered with by endogenous peaks.

Extraction recovery of vancomycin and cefazolin was determined by duplicate analyses of plasma containing 50 μg/ml vancomycin. The average peak areas obtained for each drug after taking the sample through the sample pretreatment and solid-phase extraction steps were compared to peak areas obtained by direct injection of a sample containing 50 μg/ml vancomycin and 7.5 μg/ml cefazolin. Average recovery of vancomycin was 92% and of cefazolin 83%. The solid phase sample pretreatment permits rapid sample preparation and yields good recovery. The total time for plasma sample preparation and chromatography was approximately 45 min.

All calibrations were found to be linear with no significant lack of fit over the concentration range examined at the 95% level of confidence and with coefficients of determination ranging from 0.97 to 0.99. For example, a calibration curve with triplicate samples at seven different concentration levels gave an 8.1% relative standard deviation; this statistic is based on the mean response and the variance of residuals for 21 data points and thus represents an average over the entire calibration curve. The precision of replicate analyses of the same sample was estimated using four replicate determinations of vancomycin and cefazolin in a standard plasma sample spiked with 50 μg/ml vancomycin and 7.5 μg/ml cefazolin; relative standard deviations of 0.26% for vancomycin and 1.3% for cefazolin were obtained. Day-to-day reproducibility of the method was estimated by repeating four replicate analyses of two different plasma standards (50 and 6.25 μg/ml) on two separate days. The relative standard deviations of the two concentration levels were 5.7 and 6.2%, respectively. A complete set of patient data analyzed on two different days exhibited an average difference in vancomycin concentrations of 10.7% over the six samples processed.

The concentration of each drug was not found to decrease significantly after 1 h at room temperature in the methanol–water (50:50) eluate from the Bond Elut column. Over a longer period of time, however, the cefazolin was found to degrade more rapidly than the vancomycin; for example, after 20 h at room temperature, a plasma sample spiked with vancomycin and cefazolin and processed to the methanol–water (50:50) Bond Elut eluate was found to have lost 4% of the vancomycin and 20% of the cefazolin peak area.

Interference studies were performed by injecting 100 μg of several drugs (dissolved in water or an intravenous preparation). The drugs in this specificity study were chosen either because they were coadministered during this study or were considered to be potential interferences during other vancomycin analyses. The following drugs either did not absorb at 254 nm or were not eluted from the column in a 15-min run time: digoxin, furosemide, gentamicin, nitroglycerin, phenytoin, prednisone, and prochloroperazine. Plasma (0.5 ml) was then spiked with the drugs which had been detected using the conditions described above: acetaminophen (25 μg/ml), caffeine (10 μg/ml), cimetidine (100 μg/ml), diazepam (35 μg/ml), hydralazine (40 μg/ml), procainamide (100 μg/ml), salicylic acid (25 μg/ml), and theophylline (25 μg/ml). Each of these spiked plasma samples was processed using the Bond Elut procedure and an aliquot was injected into the

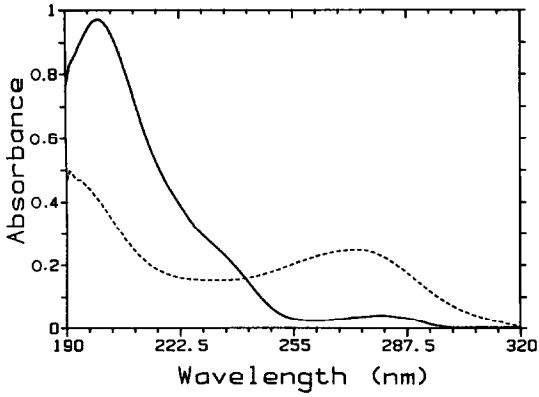

Fig. 2. UV absorbance for vancomycin (——) and cefazolin (- - - -) in the HPLC mobile phase (see text for description). The concentration of each drug was 10 μg/ml. These spectra have been corrected for the absorbance of the mobile phase.

HPLC system. Both salicylic acid and procainamide were not detected under these conditions, indicating their removal during the Bond Elut sample preparation. Acetaminophen, cimetidine, and diazepam eluted well before the vancomycin peak; the hydralazine peak eluted well after the cefazolin internal standard peak. Both caffeine and diazepam eluted just before the vancomycin peak with a relative retention (α) of 1.1, calculated relative to vancomycin; depending on the column efficiency and the doses, these two substances may interfere with vancomycin quantitation.

DISCUSSION

Predose patient plasma samples should be included in a protocol because of inter-patient plasma sample variation in endogenous peaks. Including this sample permits correction for these endogenous peaks, which might infrequently coelute with either vancomycin or the internal standard. Unfortunately, such samples were not included in the protocol for this study. In 3 out of 82 plasma samples analyzed (the final sample collected in each case), a new peak appeared that did coelute with the cefazolin internal standard. This interference was corrected by preparing the sample with and without internal standard and then subtracting out the area of the coeluting peak. Within a given patient's set of samples, additional peaks sometimes appeared which did not coelute with vancomycin or the internal standard.

In this study, vancomycin could only be quantitated in a few of the patient bone samples, although in several samples a vancomycin peak too small to integrate was present. The limit of detection of the HPLC methodology used here is approximately 3 μg/ml or 0.75 μg of vancomycin per 200-μl injection. This limit of detection might have been lowered by working at a different UV absorption wavelength, but only a fixed 254-nm detector was available for this study. Fig. 2 presents UV absorbance curves for both vancomycin and the internal standard, cefazolin. The local maximum in absorbance for vancomycin at approximately

280 nm had been previously reported [7]; vancomycin absorbance is also high around 199 nm, although the use of such a low wavelength might not be advantageous if endogenous sample materials interfere at lower wavelengths. To measure levels of vancomycin in bone adequately using our current method requires a minimum of 3 g of bone. Since the amount of vancomycin detected in the atrial appendage samples was also close to the limit of detection of the analytical method, samples of at least 0.5 g of atrial appendage are recommended.

Two additional points might indicate directions for improvements in sample preparation for this assay. First, in some chromatograms of zyphoid bone extracts in this study, early-eluting background peaks sometimes hindered quantitation of vancomycin. These early-eluting peaks might be due to zyphoid bone being porous and containing components from entrapped blood not removed in the sample clean-up. Second, it is entirely possible that appreciable amounts of drug are bound to bone and not removed by extraction. Extracted bone could be further analyzed for bound drug by demineralizing in acidic solution and cleaning up the sample with size-exclusion column chromatography to remove salts and other low-molecular-weight materials prior to reversed-phase HPLC.

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NOTE ADDED IN PROOF

Just as this article went to press, an article using a similar solid-phase extraction and reversed-phase HPLC method for vancomycin was published [9].

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