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NEW RAPID METHOD OF ANALYSIS OF CEFOXITIN IN SERUM AND BONE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the extraction and quantification of cefoxitin in blood and bone samples is described in this paper. The procedure, which prepares biological material for reversed-phase high-performance liquid chromatographic analysis is convenient, rapid and reproducible. It also allows for use of cephalothin as an internal standard in measuring serum cefoxitin levels. Conventional extraction procedures, involving use of organic solvents, generally yield drug recoveries of 60—80%. Use of Baker - 10 SPE® disposable extraction columns allowed us to consistently obtain greater than 98% recovery of both cefoxitin and cephalothin. Methods for quantification of the extracted drugs include comparison of peak ratios (for serum) or peak heights (for bone) to first-order equations obtained from regression analyses.

INTRODUCTION

Biological materials such as saliva, serum and urine have been analyzed for cephalosporin content by high-performance liquid chromatographic (HPLC) methods [1—5]. Cefazolin, cephalothin, cefoxitin, cefotaxime, cefamandole, cefuroxime and cefoperazone (T1551) concentrations have been determined in plasma and urine specimen by reversed-phase liquid column chromatography [1]. Cefoxitin has also been measured in serum samples by reversed-phase liquid chromatography [2]. Anion-exchange HPLC has been used to detect cephalothin, cefoxitin and their metabolites in whole urine [3]. Moxalactam and cefazolin levels have been determined in bone and serum samples by HPLC [4]. Microbiological assays have also been used to demonstrate the presence of cephalosporins in samples of blood, skin, soft tissue and bone [6—12].

Procedures for the extraction of cephalosporins from biological materials vary according to methods of analysis and type of sample, with recovery of the

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drugs as low as 60%. In organic solvent extraction, low yields of recovered drug may be partially attributed to unfavorable partition coefficients of the extraction solvent.

In 1981, Dupont and De Jager [5] reported the use of Sep-Pak® cartridges to obtain 97% extraction of cefoperazone from serum samples.

The present communication reports a method which was developed independently in our laboratory for the extraction of cefoxitin from serum and bone of orthopedic patients. This method serves to increase the sensitivity of HPLC and decrease the quantity of drug lost in sample preparation. The procedure includes use of Baker-10 SPE® disposable C_{18} extraction columns and increases the yield of recovered drug 38% over procedures involving extraction with organic solvents. Not only is the method sensitive, but it is rapid and reproducible.

The use of cephalothin as an internal standard in measuring cefoxitin concentration in serum samples is also reported in this paper.

EXPERIMENTAL

Reagents and materials

Sodium cefoxitin was supplied by Merck Sharpe & Dohme (West Point, PA, U.S.A.). Cephalothin was purchased from Sigma. HPLC-grade acetonitrile and methanol (Fisher Scientific) and reagent-grade acetic acid, hydrochloric acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, phosphoric acid and potassium hydroxide (J.T. Baker) were used in these studies. Baker - 10 SPE disposable columns containing octadecyl (C_{18}) packing were used throughout these experiments.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system, which included a Model M6000A solvent delivery system, an U6K injector and a Model 440 fixed-wavelength (254 nm) detector, was utilized. A Hewlett-Packard Model 3390A integrator was used. The chromatograph was equipped with a μBondapak C_{18} (10 μm particle size; 30 cm × 3.9 mm I.D.) column and a Whatman guard column (Clifton, NJ, U.S.A.) packed with Co:Pell ODS.

Filtration of solvents was carried out using a Millipore analytical filtering system (Millipore, Bedford, MA, U.S.A.).

Preliminary treatment of samples

Samples of blood and bone were provided by orthopedic patients, who were undergoing elective arthroplastic surgery.

All samples were obtained from the surgical unit at the University of Mississippi Medical Center immediately upon notification of availability and were processed as follows:

The blood samples were centrifuged. The serum layer was removed and frozen at −60° C until analyzed.

When possible, at least 3 g, wet weight, of tissue were removed from each patient sample of cancellous bone. The bone was then cut into small pieces, approximately 2—3 mm in diameter, using a rongeur. Care was taken to select

tissue free from contamination with blood. Samples were packaged individually and frozen at −60° C until analyzed.

Preparation of standards

For serum, a stock standard solution of cefoxitin (1 mg/ml) was prepared by dissolving 26.37 mg of cefoxitin in 2 ml of 1% phosphate buffer, pH 6.0 [13], and diluting to 25 ml with drug-free serum. Working standards (12.5, 25, 50, 100, 200 and 400 μg/ml) were then made by diluting the stock solution with drug-free serum.

A solution of cephalothin (500 μg/ml), prepared in the 1% phosphate buffer, was used as an internal standard for measuring cefoxitin concentration in serum samples.

For bone, a solution of cefoxitin (1 mg/ml) was prepared in distilled water. Dilutions (50, 100, 250 and 500 μg/ml) were made and a 60-μl aliquot of each solution was added to 3-g portions of cefoxitin-free bone. These samples were then incubated for 30 min in a water bath (38° C). Final cefoxitin concentrations of the standard solution were 1, 2, 5, 10 and 20 μg/g. The use of heat in preparation of bone standards eliminated the possibility of using an internal standard because the loss of cefoxitin from samples during period of incubation with cephalothin was a probability. Since an internal standard was not used, three sets of bone standards were prepared and extracted at separate times.

All standard solutions were either stored at −20° C or kept on ice when not in use.

Assay for serum cefoxitin

Extraction procedure. To 1 ml of serum was added 0.2 ml of the solution of internal standard (cephalothin) and the sample was acidified (pH < 3) by addition of 0.5 ml of 0.4 *M* hydrochloric acid. The sample was then applied to an octadecyl (C_{18}) disposable column that had been conditioned with 2 column vols. of methyl alcohol and 2 column vols. of distilled deionized water. After aspiration of the sample, the column was washed with 1 vol. of 0.1 *M* hydrochloric acid and dried under vacuum for 1 min. The sample was then eluted with 0.5 ml methyl alcohol. A 20-μl aliquot of the methyl alcohol eluent was injected into the HPLC system.

Chromatography. The mobile phase consisted of a mixture of distilled deionized water—acetonitrile—acetic acid (70:29:1). The flow-rate was set at 1.0 ml/min. The detector was set at 0.5 a.u.f.s. All separations were carried out at ambient temperature.

Quantification. First-order regression analysis was performed on data acquired from HPLC analysis of the serum standards. The *X*-coordinate values were represented by the ratio of the peak area of cefoxitin (PACF) versus peak area of cephalothin (PACP) for a known concentration of cefoxitin. The *Y* coordinates were represented by the known concentrations of cefoxitin. Cefoxitin in serum samples was quantified by comparing HPLC data of the ratio of PACF versus PACP to the first-order equation from regression analysis of the serum standards.

Assay for bone cefoxitin

Extraction procedure. After the prepared bone samples were thawed,

approximately 3 g of each sample were weighed into screw-capped conical tubes. The bone was then mixed with water in a ratio of weight of bone (g) to volume of diluent (ml) equivalent to 1:2. The mixture was shaken at 5°C for 5 h. The diluent was then removed and frozen at −20°C overnight. The procedure for extraction of cefoxitin from the diluent was the same as for the serum, but without the addition of internal standard. A 75-μl aliquot of the methyl alcohol eluent was then injected into the chromatographic system.

Chromatography. The mobile phase consisted of a mixture of distilled deionized water—acetonitrile—acetic acid (80:19:1). The flow-rate was set at 1.0 ml/min. The detector was set at 0.02 a.u.f.s. The column was operated at ambient temperature.

Quantification. The chromatograms were quantified by comparing cefoxitin peak height to the first-order equation.

First-order regression analysis on HPLC data of cefoxitin bone standards was done as for serum standards. The *X*-coordinate values were represented by the averaged peak heights from three different preparations and extractions of the known cefoxitin concentration. The *Y* coordinates were represented by known concentrations of cefoxitin. Cefoxitin concentration in bone samples was then calculated by comparing the HPLC data of the averaged cefoxitin peak heights to the first-order equation determined by the regression analysis.

RESULTS AND DISCUSSION

Preliminary extraction of cefoxitin bone standards and samples

Due to the lack of available literature on the extraction of cephalosporins from bone, studies were done to determine the period of time necessary to extract cefoxitin from bone with water. Several drug-free bone samples were incubated with 60 μl of 1 mg/ml cefoxitin to obtain 20 μg/g cefoxitin standard. Portions (3 g) of the cefoxitin bone samples were then mixed with 6 ml water and shaken on a mechanical shaker at 5°C for a total of 24 h. Aliquots of 50 μl of the eluent were removed and quantified at 2, 3, 4, 5, 6, 7 and 24 h. Samples analyzed after 5 h showed no increase in cefoxitin concentration, hence, a period of 5 h was used throughout these experiments for extraction of the drug from bone samples.

Standard solutions of cefoxitin (1, 2, 5, 10, and 20 μg/ml) in methyl alcohol were quantified concurrently with the bone extract. Recovery of cefoxitin was calculated at 56 ± 6%. Since the recoveries were low and an internal standard was not used, three different sets of bone standards were prepared, extracted and analyzed. These analyses repeatedly gave recoveries within the 56 ± 6% range. Possibly, adsorption to the bone by cefoxitin can account for this reduction of the recovery. Efforts to improve this recovery were limited by the number of cefoxitin-free bone samples which could be obtained at surgery.

Extraction of cefoxitin from serum and bone

Extraction with C_{18} disposable columns resulted in greater than 98% recovery of cefoxitin from blood and bone standards. Organic solvent extraction, using chloroform—1-pentanol, yielded a recovery of only 60—80%.

Extraction with cyano disposable columns was tried, but since cefoxitin was detected in the eluent of the sample application, the method was rejected.

Analysis of the eluent, after application of sample to the C_{18} disposable column, revealed no loss of either drug from the column; also, analysis of the acid wash eluent, revealed loss of neither cephalothin nor cefoxitin.

The rate of elution of the first methyl alcohol portion (0.5 ml) was regulated in order to maximize the elution of cefoxitin and cephalothin. An elution rate of 1 drop every 2 sec resulted in total recovery of both drugs in the first methyl alcohol elution, as was determined by absence of drug in the second methyl alcohol eluent.

Linear regression analysis

Linear regression analysis performed on serum standards provided a correlation coefficient equal to 0.99998.

Linear regression analysis performed on bone standards provided a correlation coefficient of 0.99886.

HPLC

A typical chromatogram of the sample fraction of serum (A) and bone (B) from a patient are depicted in Fig. 1.

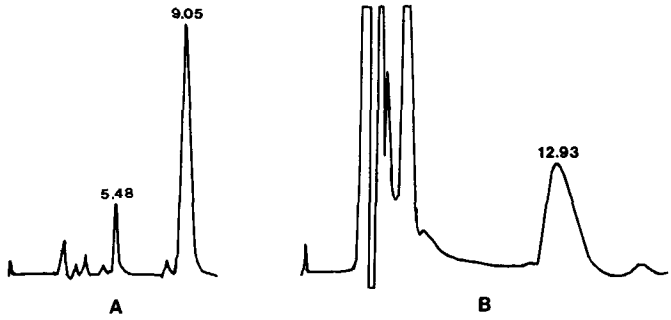

Fig. 1. (A) Chromatogram of sample fraction (0.5 ml) of extracted serum. Blood sample was drawn at time of bone excision. Cefoxitin concentration was 20.4 μg/ml. Retention time of cefoxitin is 5.48 min. Retention time of cephalothin (internal standard) is 9.05 min. λ = 254 nm; 0.5 a.u.f.s.; mobile phase: water—acetonitrile—acetic acid (70:29:1); injection volume; 20 μl. (B) Chromatogram of sample fraction (0.5 ml) of extracted bone. Cefoxitin concentration was 4.2 μg/ml. Retention time of cefoxitin is 12.93 min. λ = 254 nm; 0.02 a.u.f.s.; mobile phase: water—acetonitrile—acetic acid (80:19:1); injection volume: 75 μl.

The mobile phase was altered from water—acetonitrile—acetic acid (70:29:1) in serum cefoxitin determination to water—acetonitrile—acetic acid (80:19:1) in bone cefoxitin determination due to an unidentified peak which occurred in blank bone controls. The occurrence of this peak necessitated delaying the elution of cefoxitin in order to avoid interference. Fig. 2 shows chromatograms of extracted blank serum (A) and extracted blank bone (B) used in preparing standards. In Fig. 2B the peak at 11 min would be separated from any cefoxitin peak which would elute 2 min later, using 80% water in the mobile phase; at 70% water these two peaks are not separated.

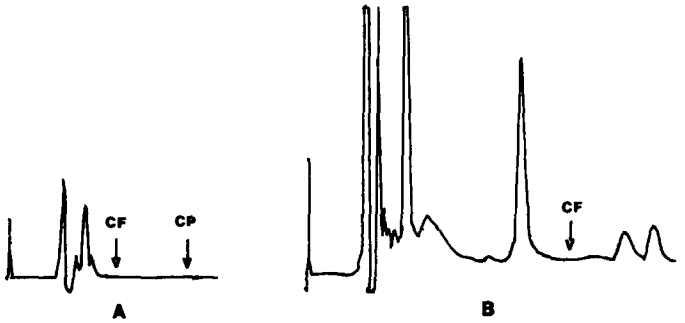

Fig. 2. (A) Chromatogram of blank serum, extracted as for serum standards and samples. HPLC conditions were the same as those in Fig. 1A. (B) Chromatogram of blank bone used in preparing bone standards, extracted as for bone standards and samples. HPLC conditions were the same as those in Fig. 1B. (↓) denotes where cefoxitin (CF) or cephalothin (CP) would elute in serum and where cefoxitin (CF) would elute in bone.

Precision

The 100 μg/ml cefoxitin serum standard was analyzed with each set of samples to determine the precision of the curve. Relative precision of 3.5% for the 100 μg/ml serum standard was obtained during the analysis of the 55 serum samples. Due to the linearity of the serum standard curve, the high rate of recoveries for cefoxitin from serum (> 98%), and the use of an internal standard, no more than one point on the serum standard curve was repeatedly extracted and analyzed.

The relative precision for the bone standard curve was 3% on the lower part of the curve and 10% on the upper portion. To compensate for the variations in precision, three different sets of bone standards were prepared, extracted and analyzed. The averages of their peak heights were used to prepare the standard curve. Bone standards were also analyzed intermittently during HPLC analysis of samples and were within the limits of the standard curve.

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