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Note

High-performance liquid chromatographic assay for piperacillin in aqueous humor of the eye

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Piperacillin is a broad spectrum, semisynthetic acylampicillin which has been shown to enter the aqueous humor and tears of the eye following intravenous administration [1]. Since it is usually effective against *Pseudomonas aeruginosa*, as well as having a broad spectrum of anti-bacterial activity, it is often used in the treatment of bacterial endophthalmitis resulting from post-surgical infections, perforating ocular injuries and corneal ulcers. With increasing frequency gram-negative bacteria such as *Pseudomonas*, *Proteus* and *Escherichia coli* have been identified as the causative organisms for intraocular infections [2–4]. It is therefore of interest to study piperacillin distribution in the eye.

Woo et al. [1] modified a high-performance liquid chromatographic (HPLC) assay for anticonvulsant drugs in order to assay piperacillin in tears and aqueous humor. They used a 193-nm detection wavelength [1]. Their assay cannot be adapted to commonly available laboratory HPLC instruments which have a 254-nm fixed wavelength detector, such as the one in our laboratory. The present paper describes an HPLC assay for piperacillin which is reproducible and sensitive enough to measure the drug at concentrations of 0.13–25.2 μg/ml in individual aqueous humor samples of 150 μl volume. The assay is a modification of that described by Brisson and Fourtillan [5] and incorporates an extraction step with chloroform–1-pentanol.

EXPERIMENTAL

Piperacillin, sodium salt, triethylamine (TEA) and morpholinopropane sulfonic acid (MOPS) were obtained from Sigma (St. Louis, MO, U.S.A.). Chlo-

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roform, HPLC grade, was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). For interference studies, drug preparations were obtained from the University of Colorado Hospital pharmacy and the Drug Assay Laboratory. Azlocillin was a gift of the Miles Pharmaceutical representative (Denver, CO, U.S.A.) and cefuroxime was a gift of the Glaxo representative (Denver, CO, U.S.A.). Methanol and 1-pentanol, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade and water was double-distilled.

An aqueous stock solution of piperacillin (252 μg/ml) was used to prepare a series of standard solutions of the drug in normal rabbit aqueous humor at a concentration range of 0.13–25.2 μg/ml. A 150-μl volume of aqueous humor was assayed. The assay was also tested using a 100-μl aliquot of aqueous humor. The working internal standard contained cephalothin at a concentration of 2.5 μg/ml.

Chromatography

A Beckman (Anaheim, CA, U.S.A.) Model 330 isocratic system, consisting of a Model 110 A pump, Model 210 sample injection valve and Model 153 detector for ultraviolet detection at 254 nm, was used in conjunction with a Hewlett-Packard (Hewlett Packard, Avondale, PA, U.S.A.) 3390 integrator-recorder. Mobile phase was pumped at 1.0 ml/min. An Altex-Beckman Ultrasphere reversed-phase octadecylsilane (RP-ODS) column, particle size 5 μm, 25 cm × 4.5 mm I.D., was wrapped in a Goldenfoil heating element. Temperature was maintained at 32 °C with a Systec temperature controller (Systec, Minneapolis, MN, U.S.A.).

The mobile phase was prepared by adding 120 ml MOPS–TEA buffer, 0.05 *M*, pH 6.7 at 30 °C, to 480 ml of distilled water. This was mixed with 400 ml of methanol. The final pH was adjusted to 6.7 (30 °C) with TEA and filtered through a 0.45-μm Millipore filter.

Assay procedure

An aliquot of aqueous humor or standard solution was placed in a 12-ml conical test tube. A 30-μl aliquot of working internal standard solution (2.5 μg/ml) was added followed by 50 μl of 0.4 *M* hydrochloric acid. These were mixed thoroughly. A mixture of chloroform–1-pentanol (3:1) (700 μl) was added and the sample mixed for 5 min by swirl-mixing and centrifuged for 5 min at 300 *g*. The aqueous layer was aspirated and discarded. A 50-μl aliquot of 0.05 *M* phosphate buffer, pH 7.0, was added to the organic layer and the sample was swirl-mixed for 5 min and centrifuged 5 min at 300 *g*. The organic layer was removed with a Pasteur pipette and discarded. The samples were centrifuged briefly and held at 4 °C until sample injection. A maximum of four samples were extracted at one time. A 20-μl aliquot was injected into the HPLC system. To obtain the concentration of piperacillin in a sample, the piperacillin/internal standard peak-height ratio of the sample was compared with the peak-height ratio of piperacillin/internal standard on the standard curve.

Precision and stability

Within-day and between-day variability was determined by analyzing ten replicate samples containing piperacillin at 2.5 μg/ml (150-μl samples) and at 5

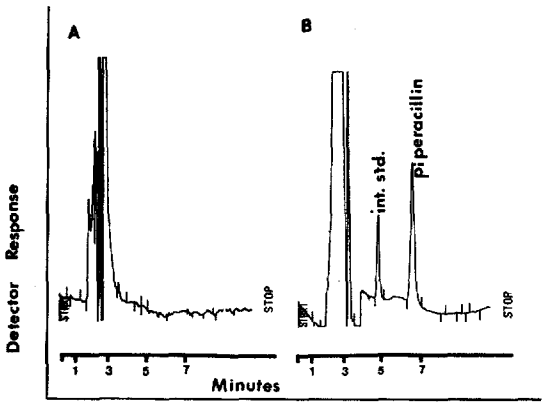

Fig. 1. (A) Chromatogram of aqueous humor obtained from untreated rabbit. (B) Chromatogram obtained by analysis of aqueous humor from a rabbit 6 h after a 50-mg subconjunctival dose of piperacillin. Concentration 4.2 μg/ml.

μg/ml (100-μl samples). Stability was verified by assaying standard solutions and the internal standard each week.

Selectivity and interference studies

Samples of aqueous humor, obtained from animals not treated with piperacillin, were analyzed without the addition of internal standard to identify potential interference by endogenous components. Interference by therapeutic agents frequently used with piperacillin, structurally similar to piperacillin, or often encountered in our patient population was evaluated (see Table I). All drugs were tested for interference at a concentration of 10 μg/ml.

Animals and drug administration

New Zealand white male rabbits (Bell Rabbitry, Clovis, NM, U.S.A.) weighing approximately 2 kg were given a 50-mg subconjunctival injection of piperacillin. After 6 h, the animals were sedated and aqueous humor was aspirated from the anterior chamber and frozen at −20°C. Samples were assayed within ten days.

RESULTS AND DISCUSSION

Chromatography

Fig. 1A is a chromatogram of rabbit aqueous humor obtained from an animal not treated with piperacillin. The results of the analysis of 100 μl of treated rabbit aqueous humor are shown in Fig. 1B; piperacillin concentration is 4.2 μg/ml. The retention time (t_R) of piperacillin is 6.86 min and of the internal standard (cephalothin) 4.74 min under the conditions described.

Assay linearity and precision

Piperacillin at a concentration range of 0.13–25.2 μg/ml in aqueous humor from untreated rabbits was assayed using 100- and 150-μl aliquots. The curves con-

structed from these standard solutions were linear. At concentrations below 0.25 μg/ml it is necessary to use the larger (150-μl) volume. Least-squares analysis yielded a coefficient of correlation (r) of 0.9973 when 150-μl aliquots were assayed; with aliquots of 100 μl, an r value of 0.9990 was obtained. Extraction recoveries were between 80 and 85%. Curves constructed from piperacillin in water were essentially identical to those in aqueous humor and were used for routine analysis.

Assaying 150-μl aliquots of standard solution, the within-day analysis of ten replicate samples containing 2.52 μg/ml piperacillin gave a mean drug concentration of 2.50 μg/ml with a coefficient of variation (C.V.) of 4.66%. The day-to-day results were 2.55 μg/ml (C.V. 4.96%). Using 100-μl aliquots at a concentration of 5.0 μg/ml, the intra-assay results were 4.85 μg/ml (C.V. 6.35%); day-to-day variation, 4.9 μg/ml (C.V. 4.42%).

Stability and interfering compounds

Piperacillin standards were stable five weeks when frozen at −20°C; cephalothin appeared to be stable for at least four months. No interference by endogenous compounds was found when aqueous humor from untreated rabbits was analyzed.

The compounds tested for interference in the assay are listed in the Table I. Chloroform–1-pentanol is a strong extraction mixture and numerous compounds are extracted. Some of these compounds show elution peaks on the chromatogram without interfering with either piperacillin or the internal standard. Retention times of these compounds are noted (see Table I). When a retention time is not listed, compounds either are eluted with the solvent front or are not extracted. Acetaminophen, mezlocillin, ampicillin, carbenicillin, salicylate and caffeine interfere with the assay. Aminoglycoside antibiotics were not tested as they do not absorb light in the ultraviolet portion of the spectrum.

This study has been directed toward aqueous humor concentrations of piperacillin following subconjunctival injections of the antibiotic. We have also used the assay to quantitate piperacillin in rabbit aqueous humor following intravenous and intramuscular administration (data not shown). With these latter techniques, approximately 1 μg/ml piperacillin was assayed in the aqueous humor 4 h after drug administration. This is consistent with the results of Woo et al. [1] who found aqueous humor concentrations exceeding 1 μg/ml for 5 h following intravenous administration of the drug to surgical patients.

Although Rouan [6] and Holdiness [7] have reviewed HPLC methods for measuring piperacillin and related drugs in plasma and urine, we found only one method for the assay of piperacillin in aqueous humor [1]. The method presented here is rapid, reproducible and selective. Under the conditions described, the limit of detection is 0.13 μg/ml of aqueous humor. The internal standard corrects for variation in recovery and injection volume. The method is precise and there are no interfering endogenous compounds. Since piperacillin is paired therapeutically with an aminoglycoside antibiotic [8], structurally related cephalosporin and penicillin antibiotics would not be present in the same sample. If salicylate, acetaminophen or caffeine are suspected, a different cephalosporin antibiotic can be substituted for the internal standard. The method is applicable to pharmacokinetic studies in animals.

TABLE I

DRUGS TESTED FOR INTERFERENCE IN CHROMATOGRAPHY OF PIPERACILLIN (t_R 6.86 min) AND CEPHALOTHIN (t_R 4.74 min)

All drugs were tested at a concentration of 10 μg/ml.

Non-interfering drugs		Interfering drugs	
Acetazolamide		Acetaminophen	(t_R 4.8 min)
Amitriptyline		Ampicillin	(t_R 4.0 min)
Atropine		Caffeine	(t_R 4.6 min)
Azlocillin	(t_R 5.4 min)	Carbenicillin	(t_R 6.8 min)
Carbachol		Mezlocillin	(t_R 6.7 min)
Cefamandole nafate	(t_R 5.5 min)	Salicylate	(t_R 4.7 min)
Cefazolin			
Cefoperazone			
Cefotaxime			
Cefoxitin	(t_R 3.0 min)		
Cefuroxime	(t_R 5.5 min)		
Chlorpheniramine maleate			
Codeine			
Diazepam			
Echothiophate iodide			
Epinephryl borate			
Imipramine hydrochloride			
Prednisolone acetate			
Scopolamine hydrobromide	(t_R 19.0 min)		
Sulfamethoxazole	(t_R 3.3 min)		
Theophylline	(t_R 3.8 min)		
Ticarcillin	(t_R 5.3 min)		
Timolol	(t_R 23.7 min)		
Tropicamide			
Xylazine			

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