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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY AND PRECLINICAL PHARMACOLOGICAL STUDIES OF PIBENZIMOL (BISBENZIMIDAZOLE)

MATTHEW M. AMES*, KRYS J. MILLER and DAVID M. MOERTEL

Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic, Rochester, MN 55905 (U.S.A.)

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SUMMARY

A sensitive and selective high-performance liquid chromatographic assay with ultraviolet or fluorescence detection has been developed for the experimental antitumor agent pibenzimol. Drug is isolated from plasma or other aqueous solutions with reversed-phase C_{18} disposable extraction columns and chromatography afforded with a deactivated reversed-phase C_{18} column and phosphate buffer—methanol mobile phase. Plasma standard curves are linear for concentrations for pibenzimol from 0.01 to 5.0 μg/ml. Pibenzimol is stable in fresh human plasma and whole blood. Pibenzimol appears to bind to plasma proteins; however, drug adsorption to glass, plastic, membranes, and filters precludes accurate determination of pibenzimol plasma protein binding. Plasma concentrations of pibenzimol fall rapidly following rapid intravenous administration to rabbits, but parent drug is detectable in plasma 24 h after drug administration. The 24-h urinary recovery of pibenzimol is 10—20%.

INTRODUCTION

Pibenzimol (bisbenzimidazole, Hoechst 33258, NSC-322921, Fig. 1) binds to adenine—thymine base pairs of double-stranded DNA and is used as a water-soluble fluorescent stain in chromosomal analysis [1—3]. It has antitumor activity in vivo against intraperitoneally implanted murine L1210 and P388 leukemia [4]. Optimal activity is observed following intraperitoneal administration on a daily X 9 schedule [4]. Pibenzimol is currently undergoing preclinical pharmacologic and toxicologic evaluation by the National Cancer Institute prior to phase I clinical trials. As part of our preclinical pharmacologic studies a sensitive and selective high-performance liquid chromatographic (HPLC) assay has been developed using fluorescence detection for the drug. The assay has

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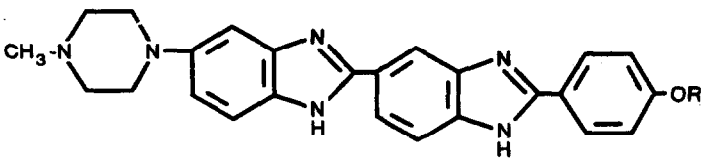

R = H (P)
R = C_2H_5 (EP)

Fig. 1. Structures of pibenzimol (P) and the O-ethyl (C_2H_5) analogue internal standard (EP).

been applied to drug stability studies, plasma protein binding studies, and rabbit pharmacokinetic studies with pibenzimol.

MATERIALS AND METHODS

Reagents

Pibenzimol was supplied as the trihydrochloride pentahydrate by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.), and also purchased from Aldrich (Milwaukee, WI, U.S.A.). The O-ethyl (C_2H_5) analogue (Hoechst 33342, Fig. 1) was purchased as the trihydrochloride from Aldrich. Amicon Centriflo cone filters and Centrifuge micropartition filter assemblies were purchased from Amicon (Danvers, MA, U.S.A.). Equilibrium dialysis chambers and dialysis membrane were purchased from Technilab Instruments (Pequannock, NJ, U.S.A.). Disposable extraction columns (C_{18}, 1 ml size) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). All solvents were glass-distilled and of chromatographic grade. All stock solutions of pibenzimol were prepared in water, methanol, dimethylsulfoxide, or mobile phase (see below) at concentrations of at least 100 μg/ml.

High-performance liquid chromatography

Analyses were performed on an IBM LC 9533 instrument equipped with an IBM Model LC 9505 autosampler. Detection was provided by a Waters 440 fixed-wavelength (340 nm) detector or a Varian Fluorichrom fluorescence detector with filters to provide excitation energy at 300—380 nm and to detect emission energy above 420 nm. The detector signal was analyzed by an IBM 9000 computer data system using the IBM CAPS software program. Chromatography was afforded by a Waters Nova-Pak C_{18} 5-μm column (15.0 × 0.39 cm) with 0.02 *M* potassium phosphate (pH 7.0)—methanol (38:62) as the mobile phase (flow-rate 1 ml/min).

Sample preparation

Aliquots of buffer, plasma, whole blood or urine (0.1—1.0 ml) were diluted to 2 ml with phosphate buffer (0.01 *M*, pH 11.0) and the internal standard added (0.5 μg in 10 μl water). Samples were applied to extraction columns which had been rinsed with 2 ml each of methanol, water, and buffer. Sample application was followed by a water wash (2 ml) and by elution with methanol

(4 ml). Methanol was evaporated under a gentle stream of nitrogen, the residue reconstituted in 100—200 μl mobile phase and 20 μl were injected for analysis. Quantitative analysis of pibenzimol was provided by comparisons of pibenzimol/internal standard peak area ratios of unknown samples to those of standard curves prepared by adding known amounts of pibenzimol (0.01—10.0 μg) and internal standard (0.5 μg) to the appropriate solution.

Pibenzimol stability studies

Pibenzimol (0.5, 1.5, 5.0 μg/ml) was incubated for various times at 4°C, 24°C, and 37°C in phosphate buffer (0.05 *M*, pH 7.4), fresh human plasma or fresh human whole blood. Aliquots were removed at appropriate times and analyzed as described above.

Pibenzimol plasma protein binding studies

Pibenzimol was added to fresh human plasma samples to provide final concentrations of 0.5, 1.5, and 5.0 μg/ml. For equilibrium dialysis chamber studies, the buffer employed was 0.02 *M* sodium phosphate (pH 7.4) in 0.9% saline. At the completion of filtration or equilibrium periods, aliquots were removed, internal was standard added, and samples were analyzed by the HPLC assay. Standard curves were prepared for all studies.

For equilibrium dialysis chamber studies, dialysis chambers (2.5 ml each side) were fitted with dialysis membranes (0.074 mm, molecular weight cut-off 6000 daltons) which had been soaked overnight in distilled water. Buffer was added to one side of the chambers, and drug—buffer or drug—plasma solutions added to the other side. Chambers were placed in a 37°C incubator for 24 h. For cone filter (molecular weight cut-off 25 000 daltons) studies, filters were soaked overnight in distilled water. Drug—buffer or drug—plasma samples (5 ml) were placed in the cones for centrifugation at 850 *g* at 37°C. Centrifugation periods were 2.5 and 4 min for drug—buffer and drug—plasma solutions, respectively, to allow approximately 30% of the sample to pass through filters into plastic collection tubes. For micropartition filter (molecular weight cut-off 30 000 daltons) studies, approximately 1.0 ml drug—buffer or drug—plasma solutions were placed in filter assemblies prior to centrifugation at 37°C. Centrifugation was accomplished at 120 *g* and 1935 *g* for the drug—buffer and drug—plasma solutions, respectively.

Rabbit studies

Male New Zealand white rabbits (2.0—3.0 kg) were administered pibenzimol (2 or 10 mg/kg in approximately 5 ml saline) by rapid intravenous administration (approximately 2 min) into a peripheral ear vein using a vein infusion set with winged adaptor (Miniset, Travenol, Deerfield, IL, U.S.A.). Blood was collected in heparinized tubes from a peripheral vein of the ear not used for drug infusion. Urine was collected using a pediatric Foley catheter (French, Size 10, Bard, Murray Hill, NJ, U.S.A.) inserted under light anesthesia into the bladder through the urethra. Collection for the following 18 h was by drip pan into glass containers.

RESULTS

Efforts to isolate and concentrate pibenzimol from aqueous solutions by extraction with organic solvents were not successful. Recoveries were variable and usually less than 50%. Satisfactory results were obtained with C_{18} disposable extraction columns when samples were applied at pH 11.0 and materials eluted with methanol. Recoveries of pibenzimol and internal standard from plasma were approximately 70% and 60%, respectively. During these studies, it was observed that pibenzimol was adsorbed on glass and other surfaces from most dilute aqueous solutions. When relatively concentrated (100 μg/ml) solutions of pibenzimol in water, buffer, methanol, or dimethylsulfoxide were sampled over time and analyzed by ultraviolet absorbance or HPLC peak area, little or no loss of parent drug was observed. However, when the same experiments were repeated at lower concentrations (0.3—10.0 μg/ml), significant loss of parent drug was observed in aqueous solutions, but not in methanol or dimethylsulfoxide solutions. Results for one set of experiments with pibenzimol at a concentration of 1.5 μg/ml are summarized in Table I. Similar results were obtained at other concentrations (data not shown). No loss of pibenzimol was observed when samples were concentrated, reconstituted in mobile phase and stored in HPLC injection vials for 24 h. Build-up and/or carry-over of pibenzimol on the HPLC system was not observed under routine assay conditions.

TABLE I

LOSS OF PIBENZIMOL IN SOLUTIONS OVER TIME

Solution*	HPLC area ($\cdot 10^{-3}$)			
	0 min	60 min	120 min	1200 min
Dimethylsulfoxide	1179	1282	1234	1137
Methanol	1537	1320	1594	1859
Mobile phase**	1304	1246	1375	1252
Phosphate buffer, 0.05 *M*, pH 7.4	470	146	117	0
Distilled water	411	192	43	16

*1.5 μg/ml final pibenzimol concentration.
**0.02 *M* phosphate buffer (pH 7.0)—methanol (38:62).

Preliminary chromatographic studies with reversed-phase columns (C_8 and C_{18}) and buffer—methanol mobile phases provided acceptable chromatograms, but with significant peak tailing. This problem was prevented by use of a deactivated C_{18} column designed for analysis of basic drugs. Chromatograms of two plasma samples, one containing the internal standard and the other containing pibenzimol and internal standard, are illustrated in Fig. 2. Sensitivity of the assay was increased five- to twenty-fold (depending on lamp and gain settings) by using fluorescence detection rather than ultraviolet absorption detection. The limit of detection under standard assay conditions was approximately 5 ng/ml. A series of ten plasma standard curves (0.01—5.0 μg/ml pibenzimol) was evaluated for linearity and inter-assay variation. The

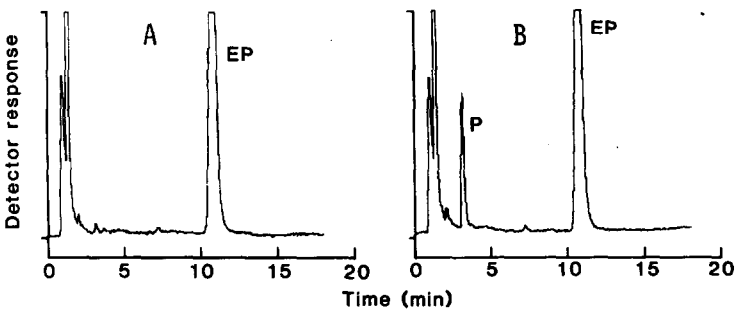

Fig. 2. Chromatograms from rabbit plasma samples (fluorescence detection) to which (A) 500 ng internal standard was added and (B) 500 ng internal standard was added and which contained 25 ng pibenzimol. Peaks: P = pibenzimol; EP = internal standard.

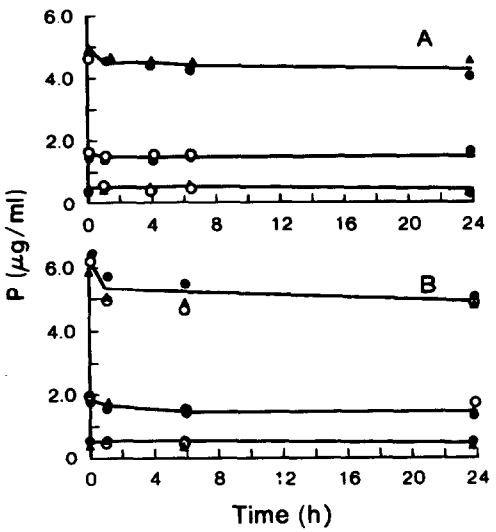

Fig. 3. Stability of pibenzimol (P) in fresh human plasma (A) and fresh human whole blood (B) at temperatures of 4°C (•), 25°C (○) and 37°C (▲).

correlation coefficient (r) was greater than 0.99 for all assays. The data were fitted to a linear model with mean slope ± S.E.M. and mean intercept ± S.E.M. values of 1.18 ± 0.03 and −0.07 ± 0.02, respectively. Coefficients of variation for concentrations of 1.0 μg/ml and 0.1 μg/ml were 4.2% and 8.7%, respectively.

Pibenzimol was stable in fresh human plasma and whole blood at concentrations from 0.5 to 5.0 μg/ml and temperatures from 4°C to 37°C (Fig. 3). There was no sequestration of pibenzimol in red blood cells. Binding of pibenzimol to human plasma proteins was studied using ultrafiltration and equilibrium dialysis chamber techniques. Control studies with buffer and drug only (in the absence of plasma) indicated adsorption of pibenzimol to filters, dialysis membranes, and dialysis chambers in that no drug was detected in ultrafiltrates and no drug crossed dialysis membranes into buffer-only chambers (Table II). There were also reduced concentrations of pibenzimol in unfiltered fractions (retentate) and in drug—buffer solutions added to dialysis chambers (Table II).

TABLE II

PIBENZIMOL PLASMA PROTEIN BINDING

Ultrafiltration and equilibrium dialysis chamber studies carried out as described in Materials and methods.

Drug concentration (μg/ml)	Percentage pibenzimol recovered			
	Equilibrium dialysis chamber		Amicon Centriflo cone filter	
	Drug chamber	Buffer chamber	Retentate*	Ultrafiltrate
Buffer studies				
0.5	0.0	0.0	47	0.0
1.5	1.1	0.0	46	0.0
5.0	3.0	0.3	27	0.0
Plasma studies				
0.5	16	0.0	68	1.3
1.5	28	0.0	69	0.0
5.0	34	0.0	57	0.0

*Recoveries corrected for reduced volume of retentate.

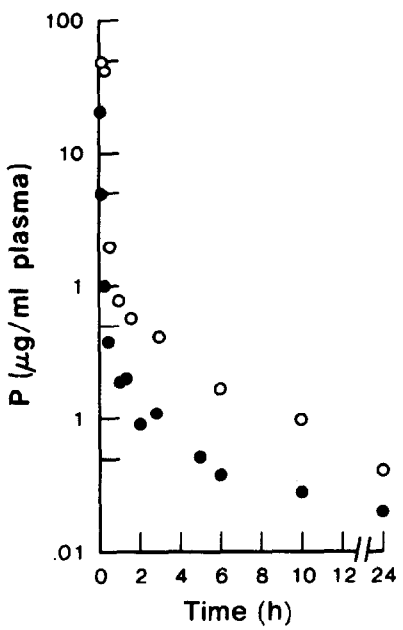

Fig. 4. Rabbit plasma time—concentration curves of pibenzimol (P) following rapid intravenous administration of 2 mg/kg (•) and 10 mg/kg (○).

Pibenzimol was detected when filters, dialysis chambers, and dialysis membranes were washed with methanol or dimethylsulfoxide and aliquots analyzed for the presence of drug. When drug—plasma solutions were substituted for buffer—drug solutions, little or no drug was detected in plasma ultrafiltrates or on the buffer side of equilibrium dialysis chambers (Table II).

However, concentrations of pibenzimol in unfiltered plasma fractions (retentate) or in the plasma—drug chambers were much higher than in the absence of plasma (Table II). Similar results for control and plasma studies were obtained when the studies were carried out with Amicon Centrifuge micropartition filters (data not shown).

Plasma concentrations and urinary recovery of pibenzimol were determined following rapid intravenous administration of 2 mg/kg and 10 mg/kg to rabbits. Plasma time—concentration curves are shown in Fig. 4. Plasma concentrations of pibenzimol fell rapidly in the first several hours after drug administration. Low concentrations of pibenzimol were detectable in plasma 10 and 24 h after drug administration. The 24-h recovery of parent drug in urine was 10—20%.

DISCUSSION

The HPLC method developed for the analysis of pibenzimol utilizes the fluorescent properties of the molecule. Fluorescence not only provides greater sensitivity than ultraviolet absorption detection, but eliminates detection of contaminating substances in plasma and urine which do not fluoresce under assay conditions. The sensitivity of the assay could be readily increased by injecting more than 20 μl of the 200 μl reconstituted sample.

The binding of pibenzimol to glass and other surfaces poses problems when working with relatively dilute aqueous solutions. When possible, methanol or mobile solvent were in contact with the drug, as in standard solutions and in the HPLC system. At concentrations used in our studies, this eliminated the adsorption problem. At even lower concentrations, others have found that dimethylsulfoxide prevents adsorption of pibenzimol to surfaces [5]. When aqueous solutions were used, concentrated stock solutions (1.0 or 0.1 mg/ml) were prepared, and small aliquots added to experimental samples. In addition, standard samples were prepared and handled in a manner identical to the experimental samples. While carryover has not been observed with HPLC injections, the least concentrated solutions were analyzed first, and blank samples were frequently analyzed as a check for this potential problem.

The loss of pibenzimol over time in aqueous solutions at concentrations of 0.3—10.0 μg/ml was not observed in stability studies with plasma and whole blood at similar concentrations of pibenzimol (0.5—5.0 μg/ml). These results suggest that pibenzimol binds to plasma proteins and other constituents of whole blood. Adsorption of drug to filters, dialysis membranes, and dialysis chambers in control studies unfortunately prevented a quantitative determination of plasma protein binding.

Following rapid intravenous infusion of pibenzimol to rabbits, pibenzimol appears to be rapidly distributed to tissues, and then more slowly eliminated as drug is still present in plasma 24 h after administration. Little parent drug is recovered in 24 h urine, owing either to metabolism and/or sequestration of pibenzimol in tissues. In preliminary studies, we have determined that pibenzimol is metabolized by rat hepatic microsomal preparations and that it is also found in the bile of cannulated rabbits [6]. Using the methodology described in this report, additional disposition and metabolism studies with pibenzimol are underway.

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