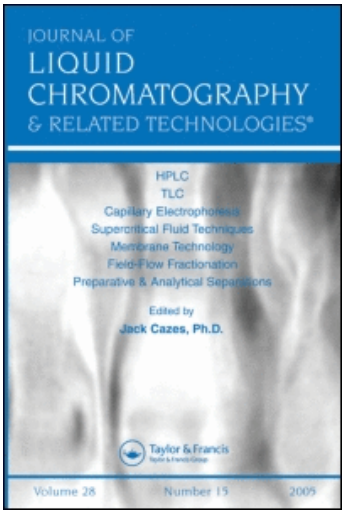

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LIQUID CHROMATOGRAPHIC ANALYSIS OF A NEW ANTIHYPERTENSIVE AGENT, PD 78,799, IN PLASMA ON SILICA GEL WITH REVERSED-PHASE ELUENT

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ABSTRACT

A sensitive and selective HPLC assay has been developed for the analysis of a new antihypertensive agent in human plasma. The drug and internal standard were isolated from plasma by liquid-liquid extraction. Separation was accomplished on unmodified silica gel with a mobile phase of 60:40 acetonitrile:10 mM dibasic ammonium phosphate. Detection was by UV absorbance at 291 nm. The method is linear over a range of 20 to 4000 ng/ml. Relative error of calibration and control standards ranged from -1.5 to 5.0% and precision, as indicated by relative standard deviation, ranged from 0.8 to 5.2%. The method has been successfully used for analysis of plasma samples from human volunteers following oral administration of PD 78,799.

INTRODUCTION

3-[[4-[4-(3-ethoxyphenyl)-1-piperazinyl]butyl]amino]-5,5-dimethyl-2-cyclohexen-1-one, PD 78,799 (Figure 1), is a new orally active antihypertensive agent. A selective assay capable of

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quantifying the drug at low nanogram per milliliter concentrations was needed for pharmacokinetic evaluation during drug development. This report describes the development and validation of an assay method employing liquid-liquid extraction of PD 78,799 from human plasma and separation by high performance liquid chromatography. An initial separation method, developed for rat and dog plasma using classic reversed-phase HPLC, was found to be subject to interferences from human plasma. The separation described here employed an aqueous-organic mobile phase on silica gel. The method has been applied to analysis of plasma samples from human volunteers receiving single oral doses of PD 78,799.

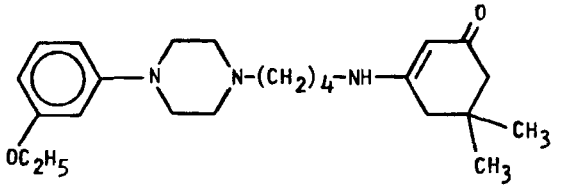
EXPERIMENTAL

Reagents

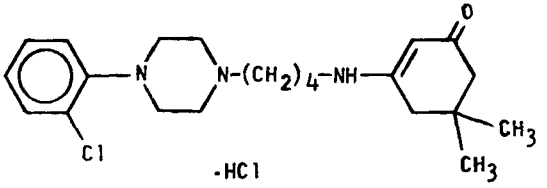
PD 78,799 and the internal standard, (IS), 3-[[4-[4-(2-chlorophenyl)-1-piperazinyl]butyl]amino]-5,5-dimethyl-2-cyclohexen-1-one, monohydrochloride (Figure 1), were synthesized at Warner-Lambert/Parke-Davis (Ann Arbor, MI). Dichloromethane and acetonitrile from EM Science (Gibbstown, NJ) and isopropanol from Burdick and Jackson (Muskegon, MI) were HPLC grade. Water was purified through a Milli-Q deionizing system (18 megaohm/cm resistivity, Millipore Corp., Bedford, MA). Sodium hydroxide, hydrochloric acid, sulfuric acid, and dibasic ammonium phosphate were reagent quality or better.

Preparation of Standards

Calibration standards containing 10, 20, 100, 200, 400, 1000, 2000 and 4000 ng/ml were prepared by adding 100 µl aliquots of a serially diluted stock solution (1.0 mg/ml) of PD 78,799 to 400 µl



PD 78,799



Internal Standard (IS)

FIGURE 1: Chemical structures of PD 78,799 and internal standard.

of blank human plasma. The 1.0 mg/ml stock solution was prepared by dissolving 10 mg of PD 78,799 in a volumetric flask containing 0.25 ml of 3 N hydrochloric acid, diluted to 10 ml with water. Quality control standards containing 100, 400, and 2000 ng/ml were prepared in blank human plasma and were frozen in 0.5 ml aliquots. Control standards were analyzed daily with calibration standards throughout validation and sample analysis periods.

The IS solution contained 200 ng per 100 µl aliquot and was prepared by dilution of an aqueous stock solution (0.2 mg/ml). Stock solutions for PD 78,799 and IS were prepared daily.

Extraction

Calibration standards, quality control standards, and 0.5 ml samples were prepared in disposable glass tubes (13 x 100 mm with

teflon-lined screw caps). To each tube 25 µl of 6.25 N sodium hydroxide and 3 ml of 3% isopropanol in dichloromethane were added. Tubes were shaken on a horizontal shaker for 15 minutes, centrifuged for 10 minutes to separate the phases, and the upper aqueous layer and any interface were aspirated. The dichloromethane phase was transferred to a clean 13 x 100 mm disposable glass tube and evaporated to dryness under nitrogen flow in a 50°C water bath. The residue was redissolved in 0.25 ml 0.002 N sulfuric acid and 0.1 ml was injected onto the column.

Chromatographic Separation

Apparatus. The HPLC system consisted of an OPG/S Gradient Mixer from Autochrom (Milford, MA); M-45 pump, 710B WISP autosampler and 480 Lambda-max UV detector set at 291 nm from Waters Associates (Milford, MA); and a 3390A Integrator from Hewlett-Packard (Avondale, PA).

Column. Separations were performed on a Brownlee MPLC 5 micron spherical silica column (220 X 2.1 mm I.D.) and matching guard column (30 mm X 2.1 mm I.D.) assembled using an MPLC cartridge system holder (Santa Clara, CA). A matching 30 X 2.1 mm I.D. precolumn placed between the pump and injector served as a presaturator. The system was operated at room temperature.

Mobile Phase. The mobile phase was acetonitrile:10 mM dibasic ammonium phosphate (60:40 v/v) mixed by the gradient mixer. Both mobile phase components were degassed by continuous helium sparging. Flow rate was 0.35 ml/min.

Plasma Recovery

Recovery from plasma was determined by adding PD 78,799 and IS to blank human plasma at concentrations of 200 and 400 ng/ml, respectively, and extracting as described above with one alteration: rather than aspirating the upper aqueous phase and

interface, two ml of the lower organic phase was drawn off with a pasteur pipet inserted through the upper aqueous phase. Mean peak heights from these extracted samples were compared to mean peak heights from the standard added to plasma.

Statistical analysis

The method was calibrated for each sample set by regressing PD 78,799/IS peak height ratios against PD 78,799 concentrations in the calibration standards. The straight line of best fit was estimated using linear regression with a weighting factor of one over concentration squared. Concentrations of PD 78,799 in unknown samples were calculated using the regression equation.

The method was validated by assaying triplicate sets of calibration and control standards on three separate days. The calibration standards from each day were fit with a straight line as detailed above. Daily calibration curves were used to back-calculate the PD 78,799 concentration of calibration and quality control standards, and these data were pooled across experimental days to evaluate reproducibility, precision and accuracy.

RESULTS

Assay Performance

Data demonstrating linearity, precision, and accuracy of calibration curves are given in Table 1. Between day reproducibility of calibration curves was demonstrated by the relative standard deviations (RSD) of the back-calculated concentrations of calibration standards. These ranged from 0.8 to 5.0% (n=9), with relative errors of -2.7 to +5.0%. A calibration curve showing data from all three validation days is presented in

TABLE 1

Precision and Accuracy Results From a Three-day Triplicate Standard Validation Study

PD 78,799 (ng/ml) Added	Found*	% Deviation From Theoretical	Relative Standard Deviation (%)
		Calibration Standards	
10	10.5	5.0	5.2
20	19.7	-1.5	3.9
100	97.3	-2.7	2.5
200	198.1	-1.0	2.6
400	401.9	0.5	1.8
1000	994.3	-0.6	2.0
2000	2023	1.2	1.4
4000	3985	0.4	0.8
		Control Standards	
100	107.4	7.4	2.0
400	412.9	3.2	2.9
2000	2042	2.1	2.9

* Mean of 9 values pooled from three separate calibration curves
% Deviation = (Found - Added)/Added x 100%

Figure 2. These results indicate that the method is linear over the range of 10 to 4000 ng/ml.

Precision and accuracy were determined from analysis of quality control standards. Assay precision was +/- 2.9%, based on RSD values (n=9) of 2.0 to 2.9% for the back-calculated concentrations of quality control standards. The accuracy of the method was +/- 7.4%, with relative errors ranging from 2.1 to 7.4% (Table 1).

Recovery

Mean extraction recovery (n=6) was 98.4% from PD 78,799 samples at 200 ng/ml and 92.7% from IS samples at 400 ng/ml.

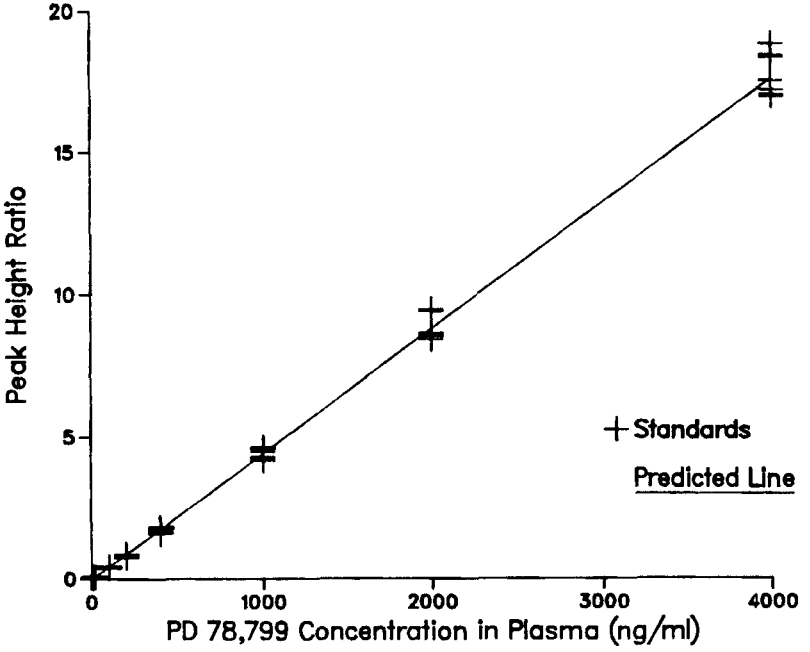


FIGURE 2: Calibration Curve showing PD 78,799/IS peak height ratios of calibration standards vs. PD 78,799 concentrations. Predicted line is drawn from slope determined using linear regression.

Application

The method was used to assay PD 78,799 concentration in plasma samples from human volunteers receiving single oral doses of the drug. Figure 3 shows plasma concentration-time profiles from two subjects following oral administration of 40 mg. Representative chromatograms from the analysis are shown in Figure 4.

DISCUSSION

The guard column in this system functioned as an easily replaceable column front. By replacing the first cartridge upon

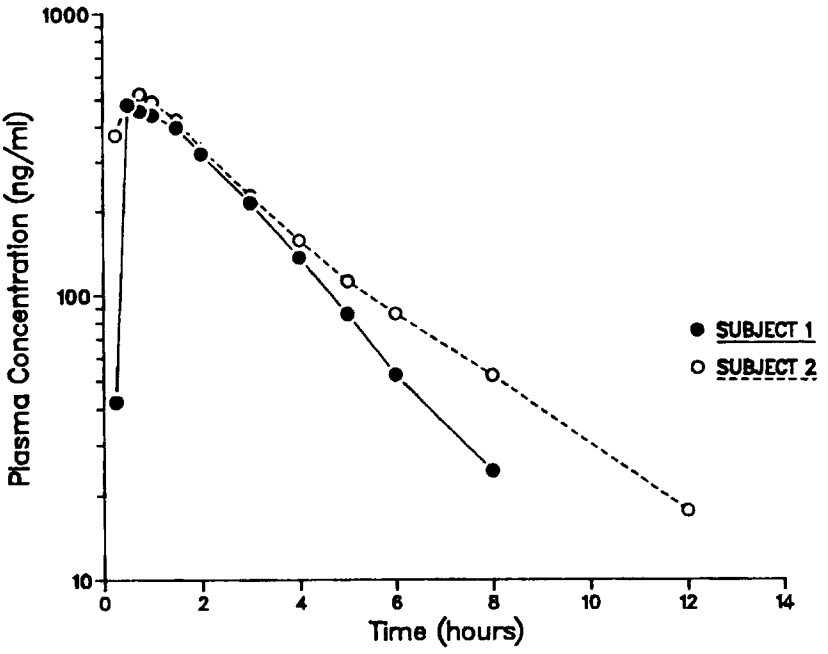


FIGURE 3: PD 78,799 plasma concentrations in healthy human volunteers following peroral administration of a 40 mg dose.

increases in pressure or decreases in resolution, analytical columns have been used for as many as 1000 plasma samples. In general, the guard column was replaced every 200 to 300 samples. The matching presaturator column protected the microparticulate silica analytical column from erosion during the long-term use of an alkaline semi-aqueous mobile phase (apparent pH 7.9). In addition, use of a 2.1 mm I.D. column yielded an approximate 4-fold increase in peak height due to reduction in the peak volume, yet no special equipment or detector flow cells were necessary [1].

An initial separation for PD 78,799 employing reversed-phase HPLC on a Brownlee RP-18 column (C18, monofunctional, 5 micron

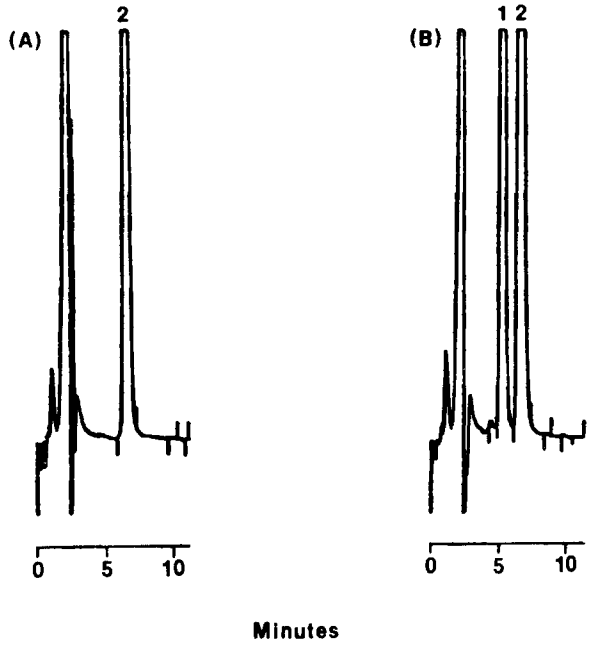


FIGURE 4: Chromatograms from the analysis of PD 78,799 in plasma from human volunteers before (A) and 1.5 hours after (B) peroral administration of a 40 mg dose. Conditions given in text. 1 = PD 78,799; 2 = internal standard.

spherical) was developed using rat and dog plasma and validated in human plasma. When this method was applied to plasma from human subjects enrolled in a pharmacokinetic study, however, a major interference peak was observed at the retention time of PD 78,799 (Figure 5). A related compound had been successfully analyzed using an aqueous-organic mobile phase on a Brownlee 5 micron spherical silica column [2]. Therefore, a similar separation was developed for PD 78,799 which proved to be completely free of the interference observed on the C18 column.

A specific and selective separation was provided by the strong interaction of PD 78,799 with unmodified silica gel in an aqueous-organic mobile phase. This type of interaction,

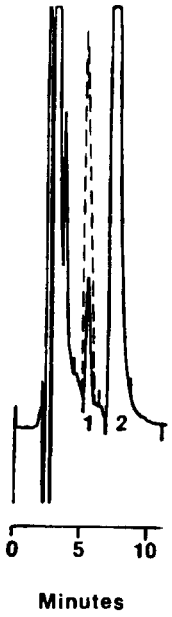


FIGURE 5: Chromatogram of a plasma calibration standard (20 ng/ml) on a Brownlee RP-18 column (C18, monofunctional, 5 micron spherical), 2.1 mm I.D. x 220 mm with matching 2.1 mm I.D. x 30 mm guard cartridge. Dashed line indicates appearance of interfering peak found in predose plasma samples from human volunteers. 1 = PD 78,799; 2 = internal standard. Mobile phase is 70:30, methanol:20 mM dibasic ammonium phosphate. Integrator set at 8 mV full scale with 1 mV/mA detector output.

attributed to an ion-exchange interaction between the drug and surface silanol groups, has been reported by Bidlingmeyer et al. for a variety of lipophilic amine drugs [3]. Similar methods have been successfully used for assay of several drugs [4,5,6]. Although drug elution patterns for these systems are similar to those of alkyl-bonded reversed-phase columns, entirely different selectivities may be offered for separation of interfering plasma components. In this instance, a sensitive, precise and accurate method for the determination of PD 78,799 in human plasma was developed which was free of plasma interferences observed on the C18 column.

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