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AUTOMATED CAPILLARY GAS CHROMATOGRAPHIC ASSAY USING NITROGEN—PHOSPHORUS IONIZATION DETECTION FOR THE DETERMINATION OF BEPRIDIL IN HUMAN PLASMA

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SUMMARY

A highly sensitive and specific capillary gas chromatographic method for the determination of the antianginal drug bepridil in plasma is described. The capillary gas chromatograph and nitrogen-selective detector combination provides excellent sensitivity for clinical samples. The lowest concentration of bepridil which can be measured accurately and precisely in a 1-ml plasma sample is 1 ng/ml. Standard curves are linear over the concentration range 1—60 ng/ml. Accuracy and precision of the assay, expressed as relative deviation from the true value and relative standard deviation (inter-run) are < 15% at all concentrations in the linear range. No interfering peaks are observed. Using an automatic injector and a laboratory computer system, sixty samples can be analyzed routinely in one day. The present assay has been successfully cross-validated with a published high-performance liquid chromatographic assay.

INTRODUCTION

Bepridil hydrochloride, β-[(2-methylpropoxy)methyl]-N-phenyl-N-(phenylmethyl)-1-pyrrolidineethanamine monohydrochloride monohydrate, is a calcium-channel blocker currently under clinical evaluation for the treatment of angina pectoris. Effects on cardiovascular parameters have been evaluated in animal studies [1—4] and its antianginal properties have been demonstrated in clinical studies [5, 6].

Therapeutic doses of bepridil hydrochloride (300—400 mg) usually yield plasma bepridil concentrations in the 10—1000 ng/ml range. Plasma concentrations in this range are quantifiable using the high-performance liquid chromato-

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graphic (HPLC) assay of Ng et al. [7] or the packed-column gas chromatographic (GC) assay of Vink et al. [8]. The HPLC assay has a quantitation limit of 10 ng/ml using 2 ml of plasma and the GC assay has a quantitation limit of 5 ng/ml using 2–3 ml of plasma. However, in situations where limited sample is available, these assay procedures are inadequate. Additionally, since bepridil plasma concentrations after administration of a single oral dose may fall below 10 ng/ml after 24 h in some subjects, a more sensitive assay was required to fully characterize the elimination kinetics of bepridil in these subjects.

The present study reports the development of a sensitive and reproducible capillary GC assay with nitrogen–phosphorus ionization detection (NPD) for bepridil in plasma. The assay was cross-validated with the published HPLC procedure [7] using plasma samples from healthy volunteers and was also used to examine the elimination kinetics of bepridil in subjects with plasma bepridil levels less than 10 ng/ml at 48 h after a single 400-mg oral dose of bepridil hydrochloride.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard 5880A capillary gas chromatograph equipped with a Hewlett-Packard 7672A autosampler and a nitrogen–phosphorus ionization detector was used. A CP Sil 8 CB fused-silica capillary column (25 m × 0.32 mm I.D.; 0.12 μm film thickness, Chrompack, Bridgewater, NJ, U.S.A.) was used with helium as carrier gas at a flow-rate of 3 ml/min at 190°C. The injector and detector temperatures were 300°C and oven temperature programming was employed from 190 to 255°C at 15°C/min. Splitless injection with a purge at 0.3 min was used in conjunction with the autosampler.

A Hewlett-Packard 3354 Laboratory Automation System with software developed in-house was used for data acquisition and processing.

Reagents and supplies

Nanograde methanol, toluene and hexane were obtained from Mallinckrodt (Paris, KY, U.S.A.) and used without further purification. Triply purified distilled water was obtained from Ephrata Mountain Water (Manheim, PA, U.S.A.).

Glacial acetic acid and ammonium hydroxide (58%), analytical-reagent grade, were purchased from Mallinckrodt and HPLC-grade ammonium acetate was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

C_{18} Bond-Elut cartridges, 500 mg capacity, and the Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Bepridil hydrochloride and the internal standard, a bepridil analogue, were obtained in-house (McNeil Pharmaceutical, Spring House, PA, U.S.A.). Structures for these compounds are given in Fig. 1. All bepridil concentrations in this report refer to the free base unless otherwise noted.

Extraction procedure

To a 1-ml plasma sample, 2 ml of methanol containing 20 ng/ml internal

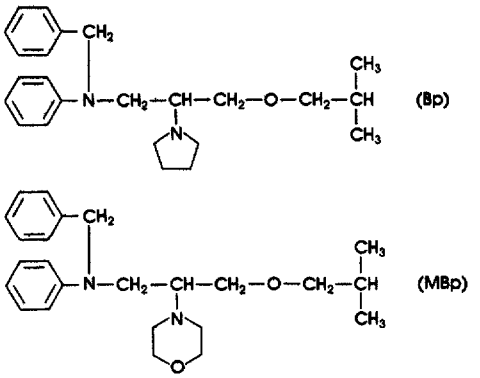

Fig. 1. Chemical structures of bepridil (Bp) and the internal standard used in this assay (MBp).

standard were added to precipitate the plasma proteins. After vortexing and centrifuging (at 681 g) the mixture, the supernatant was decanted into the reservoir of a conditioned C_{18} Bond-Elut cartridge (cartridges were conditioned by washing with 3 ml of 0.04 *M* ammonium acetate in methanol and 6 ml of methanol). Following the application of the sample, the cartridge was rinsed with 3 ml of 1.5 m*M* acetic acid once and 3 ml of methanol—water (75:25, v/v) twice.

The sample was eluted using three 0.5-ml aliquots of 0.03 *M* ammonium acetate in methanol. The eluent was transferred to a centrifuge tube containing 8 ml of hexane and 100 μl of 0.58% ammonium hydroxide, and the tube was vortexed for 10 s. After allowing the layers to separate, the hexane layer was transferred to another tube and evaporated using a gentle nitrogen stream.

The dried residue was reconstituted with 50 μl of a toluene—methanol (90:10, v/v) solution and the sample was transferred to autoinjector vials. A 3-μl aliquot was injected into the capillary gas chromatograph for analysis.

Standard curves

To establish a calibration curve, a series of bepridil standard solutions (1—60 ng/ml) containing 20 ng/ml internal standard were prepared in methanol using silanized glassware. A 2-ml volume of these solutions was added to 1 ml of plasma (instead of the 2 ml of methanol containing internal standard alone) and the samples were extracted according to the procedure above. Duplicate standard curves were run on each analysis day. The peak-height ratios of bepridil and the internal standard were weighted by 1/variance and plotted against the bepridil concentrations. Linear regression analysis gave a calibration line which was used to calculate bepridil concentrations in unknown samples and frozen seeded controls.

As an additional control, seeded plasma pools were prepared at three concentrations (5, 20 and 40 ng/ml bepridil), separated into 1-ml aliquots and frozen. Two samples from each pool were analyzed with each calibration curve to assess the precision of the assay procedure.

Assay validation

Bepridil plasma concentrations were determined in over thirty samples from

clinical trials using both the published HPLC procedure [7] and the present assay. For samples that had concentrations above the linear range of the GC assay, smaller aliquots of plasma (50 μl, 100 μl and 0.5 ml) were diluted to 1 ml with distilled water prior to analysis. The data sets were compared to determine their correlation coefficient, and the slope and intercept of the corresponding regression line. Additionally, plasma concentration versus time profiles from subjects with plasma levels < 10 ng/ml after 24 h were analyzed to verify the utility of the assay in characterizing the elimination kinetics of bepridil.

RESULTS AND DISCUSSION

Gas chromatography

Chromatograms of plasma with and without bepridil and the internal standard are shown in Fig. 2. The retention times of bepridil and the internal standard are 4.53 and 5.18 min, respectively. No significant interfering peaks appear in the chromatogram of blank plasma and the two compounds are well separated.

Additionally, owing to the selectivity of the cartridge procedure and the resolution of the capillary column, none of the following drugs interfere with the determination of bepridil in plasma: amitriptyline, diazepam, phenobarbital, aspirin, acetaminophen, ibuprofen, chlorothiazide and propranolol.

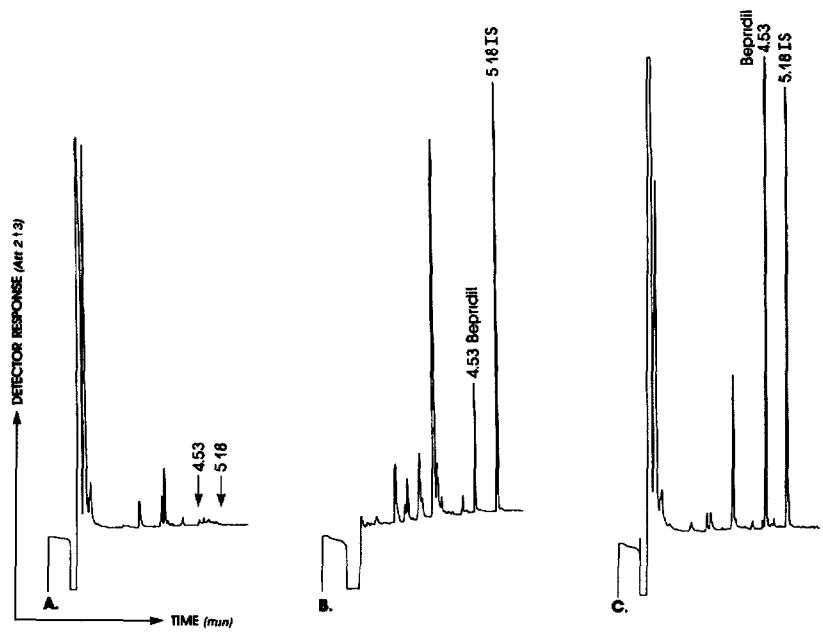

Fig. 2. Capillary GC—NPD profiles of (A) blank plasma; (B) plasma spiked with 15 ng/ml bepridil and 40 ng/ml internal standard (IS); and (C) a clinical plasma sample containing 82 ng/ml bepridil (0.5 ml was analyzed).

TABLE I

SUMMARY OF STANDARD CURVE DATA FOR THE ANALYSIS OF BEPRIDIL IN PLASMA

Duplicate standard curves analyzed on three separate days.

Concentration seeded (ng/ml)	n	Mean concentration measured (ng/ml)	Relative standard deviation (%)	Mean deviation from seeded concentration (%)
1	6	1.10	10.0	9.7
3	6	2.59	8.3	13.8
5	6	4.98	14.7	0.5
10	6	9.6	9.8	4.3
15	6	14.2	7.3	5.3
20	5	20.2	12.3	1.0
30	6	32.2	11.5	7.8
40	6	43.6	7.1	9.0
60	6	64.0	6.5	6.6

Day	Linear regression analysis		
	Slope	y-Intercept	Correlation coefficient
1	0.021	0.001	1.00
2	0.022	—0.011	1.00
3	0.023	—0.019	1.00
Three-day composite	0.022 ± 0.001	—0.002 ± 0.001	0.99

Standard curves, precision and accuracy

Duplicate calibration curves run on three consecutive days were linear over the concentration range studied here (1—60 ng/ml) (Table I). Regression analysis of the peak-height ratio data gave the following equation: $y = 0.021x - 0.002$ (y = peak-height ratio of drug to internal standard; x = bepridil concentration). The correlation coefficient for the three-day composite curve was 0.99. The precision of the assay, as measured by the relative standard deviations at each concentration, was within 15%. The average back-calculated concentration was within 10% of the seeded value at each concentration except at the 3 ng/ml level (< 14%). The average measured concentrations of the frozen seeded control plasma samples were within 14% of their theoretical (spiked) concentrations with relative standard deviations of less than 14%.

Recovery and stability

The extraction efficiency for bepridil was determined using the ^{14}C-labeled compound at concentrations of 4 and 50 ng/ml. The plasma extraction efficiencies at these concentrations were 55.1 ± 1.6% and 50.2 ± 3.3% at 4 and 50 ng/ml, respectively ($n = 6$). The extraction efficiency of the internal standard was determined to be 50.9 ± 3.6% at 40 ng/ml ($n = 6$). Bepridil has been shown to be stable in frozen plasma for at least one month [7]. Additionally, the dried plasma extracts are stable overnight and may be injected the next day.

Assay validation

Bepridil plasma concentrations were determined in each of the human

samples using both the HPLC procedure [7] and the capillary GC—NPD assay. The results from each assay are presented in Fig. 3. The correlation coefficient for the two data sets was 0.99 and the corresponding regression line weighted by 1/concentration gave a slope of 0.98 ± 0.02 with a y-intercept of −1.6 ± 6.3 ng/ml.

Fig. 4 illustrates the plasma concentration versus time profile of a subject obtained with both the HPLC and capillary GC—NPD procedures. Using the capillary GC assay, bepridil plasma concentrations were measurable up to 120 h following the administration of a single oral dose of 400 mg of bepridil hydrochloride. The HPLC assay, however, was only able to measure bepridil concentrations up to 48 h following dose administration. The increased sensitivity of the capillary GC assay allows more accurate calculation of the terminal elimination half-life of bepridil in such subjects.

In summary, a capillary GC—NPD assay for bepridil in plasma has been

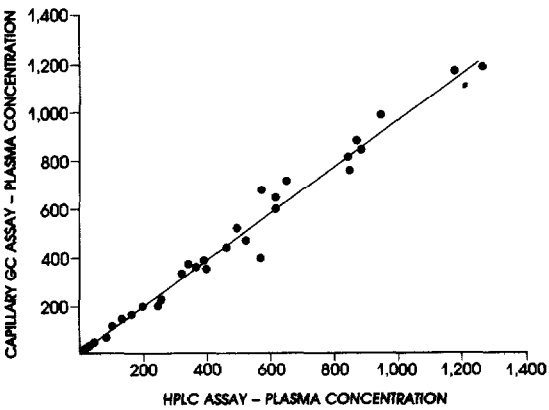

Fig. 3. Data points (ng/ml) and the regression line for the comparison of HPLC and capillary GC assay results. Regression analysis gave a slope of 0.98 with a y-intercept of −1.6 ng/ml. The correlation coefficient was 0.98.

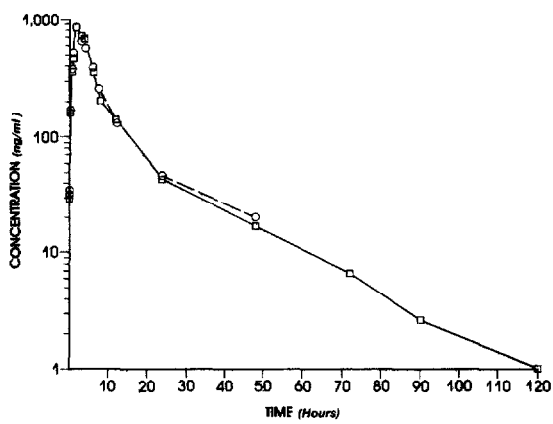

Fig. 4. Plasma concentration versus time profile obtained from a human subject following the administration of a single oral dose of 400 mg bepridil hydrochloride. ○, Capillary GC assay results; □, HPLC assay results.

developed that is capable of measuring as little as 1 ng of bepridil in 1 ml of plasma or up to 1200 ng/ml using 50 μl of plasma. The assay complements the available HPLC assay by allowing smaller sample sizes to be utilized and by lowering the quantitation limit for bepridil in plasma to 1 ng/ml. Additionally, the assay has demonstrated utility in measuring bepridil concentrations in subjects with plasma bepridil levels less than 10 ng/ml at 48 h after a single 400-mg oral dose of bepridil hydrochloride.

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