An LC Method for Quantifying Bepridil in Human Plasma Using 1-Naphthol as the Internal Standard

Kumiko Noda[1], Yasuhiro Narayama[1], Yoshikazu Goto[2], Michiya Kobayashi[1], Hiroshi Kuronuma[2], Shinji Iwai[2], Katsumi Itoh[2], Noriyoshi Katoh[3], and **Koji Tadano[1,]***

[1]Department of Practical Pharmacy, Faculty of Pharmaceutical Science, Health Sciences University of Hokkaido, [2]Pharmacy, and [3]Cardiovascular Medicine, Sapporo City General Hospital, Sapporo, Japan

Abstract

A modified method for the quantitative determination of bepridil hydrochloride in human plasma is described. This method is unrelated to chemical methods currently in use. The mobile phase is 50 mM phosphate buffer (pH3.0)–methanol–acetonitrile–triethylamine (57:3:40:1, v/v), and the samples are fractionated on a C8-3 column (150 × 4.6 mm, 5 µm) using a flow rate of 0.9 mL/min. Bepridil was detected by UV spectroscopy at 254 nm. The retention times of bepridil and 1-naphthol were 12.6 min and 7.5 min, respectively; there was no interference originating from human plasma. We confirmed that the bepridil and 1-naphthol peaks were not influenced by the presence of 32 commercial medicines frequently co-administered with bepridil. Additionally, the concentration of bepridil in the plasma of five patients treated with bepridil for atrial fibrillation was measured. These samples were collected just before each dosage of bepridil. Their rhythm and rate control were well maintained. Trough concentrations ranged from 233.9 to 347.4 ng/mL, similar to previously reported values.

Introduction

Bepridil hydrochloride, β-[(2-methylpropoxy)methyl]-*N*-phenyl-*N*- (phenylmethyl)-1-pyrolidineethanamine monohydrochloride (Figure 1), is a calcium-channel blocker that can affect cardiovascular activity. Recently, several Japanese groups have reported beneficial effects of bepridil in the treatment of patients with atrial fibrillation (AF) (1–3). Bepridil was indicated as a drug for treatment of persistent AF in Japan in 2008. However, the required plasma concentration of bepridil has not been verified, and a major safety concern with bepridil is the occurrence of ventricular arrhythmias, especially torsades de pointes-associated QT interval prolongation (1). Clarification is thus needed regarding whether therapeutic drug monitoring of bepridil concentrations in plasma is useful for evaluating fibrillational efficacy and the occurrence of adverse effects. This would require measuring the concentration of bepridil in plasma clinically. Current methods for measuring plasma bepridil concentrations are difficult, as they require an internal standard (IS) obtained from companies supplying bepridil for clinical use (4,5), while methods using reverse-phase thin-layer chromatography or gas chromatography for routine clinical use lack quantifiability or have limited applicability (6,7). The purpose of this study was to develop a method for assaying plasma concentrations of bepridil using easily obtainable materials, and to measure the concentration of bepridil in the plasma from patients with AF. Patients with AF are clinically co-administered medicines with bepridil. During therapeutic drug monitoring, peaks from co-administered medicines frequently disturb quantity measurements of the target drug. Therefore, 32 medicines commonly co-administered with bepridil were also investigated to determine whether they interfere with the quantification of bepridil using this modified quantitative method.

* Author to whom correspondence should be addressed: Department of Practical Pharmacy, Faculty of Pharmaceutical Science, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobetsu Hokkaido 061-0293, Japan, email: tadano-k@hoku-iryo-u.ac.jp.

Experimental

Chemicals

Bepridil hydrochloride was purchased from Alexis Biochemicals (Farmingdale, NY). 1-Naphthol, triethylamine, phosphoric acid, sodium hydroxide, HPLC grade acetonitrile, methanol, and hexane were purchased from Wako Biochemicals (Osaka, Japan).

Patients and ethics

The study protocol was approved by the institutional review board at Sapporo City General Hospital, and all patients gave written informed consent. Five patients were identified which

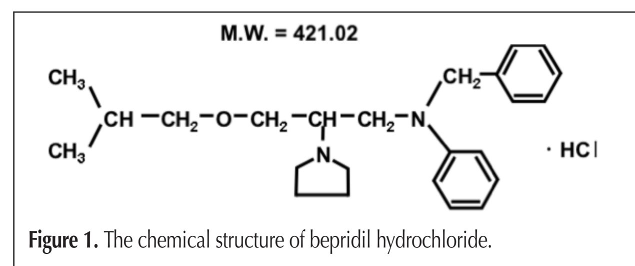

Figure 1. The chemical structure of bepridil hydrochloride.

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had already been treated orally for one to two years with bepridil (100–200 mg/day) for the control of AF. These patients stopped taking oral bepridil on the morning of the test day, and blood samples were collected before each dosage.

Plasma collection and processing

Blood samples were collected from the patients in heparinized test tubes. The samples were centrifuged at 400 × g for 20 min at 25°C. The plasma fractions were removed and stored at −20°C until assayed.

Sample preparation

Phosphate buffer (0.5 mL, 100 mM, pH 7.4), 0.5 mL IS solution (2.5 µg/mL 1-naphthol dissolved in acetonitrile) and 0.5 mL acetonitrile with and without a standard concentration of bepridil (for standard curve samples and samples from patients taking bepridil, respectively) were added to 0.5 mL plasma sample. Bepridil and IS were extracted with 2.5 mL *n*-hexane for 10 min at 200 rpm using a 5415R centrifuge (Eppendorf, Hamburg, Germany). The *n*-hexane layer was transferred to a glass tube. *n*-Hexane (2.5 mL) was added again to the residue and the extraction step was repeated. The total *n*-hexane layer (5 mL) was evaporated at 37°C using a TC-8 concentrator (Taitec, Saitama, Japan). The residue was dissolved in 0.25 mL mobile phase, and injected onto the HPLC system.

Chromatographic conditions

A HPLC system (Shimadzu, Kyoto, Japan) with a pump (LC-10A), column oven (CTO-10AS), a UV detector (SPD-10A) and an Inertsil C8-3 column (150 mm × 4.6 mm i.d.; 5.0-µm particle size; GL Sciences, Tokyo, Japan) was used. Data acquisition was performed with an integrator (C-R8A). The mobile phase was comprised of 50 mM phosphate buffer (pH 3.0)–methanol–acetonitrile–triethylamine (57:3:40:1, v/v), based on previous methods (4,5). The flow rate was 0.9 mL/min and the temperature of the column oven was maintained at 55°C. The peaks were monitored by UV (wavelength: 254 nm).

Assay validation

Selectivity and specificity of the method were determined by examining four different plasma samples for any interfering peaks at the retention times corresponding to bepridil and 1-naphthol.

Linearity

Daily calibration curves were obtained using five standard concentrations of the drug (25, 100, 250, 500, and 1000 ng/mL). A blank sample was also included in each series. Each standard was prepared using 500 µL blank plasma with 20 µL each of working acetonitrile solution at concentrations that were 25-fold higher than those intended for the standards. The nominal concentra-

Table I. Retention Time and Effect of Extraction for Cardiovascular Medicines Frequently Co-administered to Patients Taking Bepridil

			Retention time (min)	
	Chemical name	Commercial name*	Before extraction	After extraction
	Bepridil hydrochloride	Bepricor	12.6	12.6
	hydrate 1-Naphtol (IS)	–	7.5	7.5
Calcium channel blocker				
	Amlopdipine besylate	Amlodin	3.9	–
	Cilnidipine	Atelec	No peak	–
	Nilvadipin	Nivadil	No peak	–
	Verapamil hydrochloride	Vasolan	4.2	–
	Diltazem hydrochloride	Herbesser	3.0	–
ARB (Angiotensin II receptor inhibitor)				
	Olmesartan medoxomil	Olmetec	10.3	–
	Valsartan	Diovan	13.4	–
	Candesartan cilexetil	Blopress	No peak	–
	Losartan potassium	Nu-Lotan	8.0	No peak
Anti-platelet agents				
	Aspirin	Bufferin	3.5	–
	Clopidogrel sulfate	Plavix	No peak	–
	Warfarin potassium	Warfarin	13.9	No peak
	Ticlopidine hydrochloride	Panaldine	10.8	–
Antidiabetic agents				
	Glimepiride	Amaryl	No peak	–
	Gliclazide	Glimicron	13.6	No peak
	Metformin hydrochloride	Glycoran	1.4	–
Beta-blocker				
	Bisorolol fumarate	Maintate	No peak	–
	Propanolol hydrochloride	Inderal	2.5	–
Diuretics				
	Furosemide	Lasix	4.6	–
	Trichlormethiazide	Fluitran	3.9	–
Inotropic agents				
	Digoxin	Digosin	No peak	–
	Methyldigoxin	Lanirapid	6.9	–
Anti-aginal agents				
	Isosorbide mononitrate	Itorol	No peak	–
	Nicorandil	Sigmart	2.6	–
Others				
	Enalapril maleate	Renivace	No peak	–
	Doxazosim mesilate	Cardenalin	No peak	–
	Amenzinium methylsulfate	Risumic	10.0	–
	Rosuvastatin calcium	Crestor	8.4	No peak
	Pitavastatin calcium	Livalo	10.7	–
	Allopurinol	Zyloric	1.6	–
	Fursuliamine hydrochloride	Alinamin F	2.0	–
	Pranlukast hydrate	Onon	No peak	–

* Commercial names were approved in Japan

tion of the standard is represented as X below. The concentration of bepridil in the samples was calculated from the following equation using appropriate regression analysis:

$$\text{Bepridil concentration } (\mu g/mL) = \alpha \times X + \beta$$

where X is the peak area ratio of bepridil/1-naphthol, α is the slope of the calibration curve and β is the intercept of the calibration curve.

Limit of detection and quantitation

Using the calibration curve constructed from the standard solutions, the limit of detection (LOD) was defined as the lowest concentration that can be detected but not necessarily quantified, and the limit of quantitation (LOQ) was defined as the lowest concentration that can be determined with acceptable accuracy and precision.

Accuracy and precision

Accuracy was determined as the absolute value of the ratio of the back-calculated mean values of the quality control (QC) samples (25, 250, and 1000 ng/mL) to their respective nominal values, and expressed as a percentage.

Precision of the assay was measured by the percent coefficient of variation (CV%) over the concentration range of low, medium and high QC samples (25, 250, and 1000 ng/mL, respectively) of bepridil during the course of validation, and were determined from nine consecutive preparations (within-day) and four independent assays from one experimental day (between-day).

Selectivity

Thirty-two commercial medicines (Table I) frequently co-administered with bepridil to patients were tested to determine whether they interfered with the bepridil and IS peaks on HPLC. These medicines were dissolved in the mobile phase and injected onto the HPLC system. Medicines which produced a peak that interfered with the quantification of bepridil or the IS were added to a mixture including 0.5 mL of 100 mM phosphate buffer (pH 7.4), 0.5 mL acetonitrile and 0.5 mL plasma sample (drug free), and then extracted with *n*-hexane. The following step was in accordance with "Sample preparation."

Stability of analytical solutions

Long-term stability of the drug in plasma was tested by comparing low, medium and high QC samples stored at –20°C for 42 days with that of freshly prepared samples. The percent degradation was determined by comparing the mean of back-calculated concentrations of bepridil from three frozen samples with that of freshly thawed QC samples.

Results and Discussion

Optimized chromatographic conditions

Chromatographic conditions were based on the method reported by Taguchi et al. (5), which used a mobile phase comprised of 10 mM KH_2PO_4 containing 2% (w/v) triethylamine–acetonitrile (60:40, v/v) and a column packed with C-18 (150 mm × 4.6 mm i.d.; 4.5-µm particle size). Alternatively, we used a C-8 column, as bepridil was undetectable using the C-18 column. Moreover, we modified the mobile phase by changing the buffer and applying acetonitrile instead of methanol to avoid interference from plasma-originating peaks.

Selectivity and specificity

Representative chromatograms of blank plasma (Figure 2A) and plasma spiked with 250 ng/mL bepridil and 2.5 µg/mL 1-naphthol in acetonitrile as the IS (Figure 2B) are presented. No interference was observed in blank plasma samples in the vicinity of the bepridil and IS peaks. The fractions from plasma samples corresponding to peaks with retention times of 5.3 min and 6.7 min and plasma-related noise around 10 min were separated from those for bepridil (12.5 min) and the IS (7.5 min). 1-Hydroxy-2-naphthoic acid, ethyl *p*-aminobenzoate and butyl *p*-aminobenzoate were also examined for their use as an IS. In the case of 1-hydroxy-2-naphthoic acid, impurities were found near the peak for bepridil, and in the cases of ethyl *p*-aminobenzoate and butyl *p*-aminobenzoate, their peak retention times were approximately 10 min, within the noisy period originating from human plasma, making them unsuitable as an IS. Blank plasma spiked with several commercial medicines did not appear to interfere with peaks originating from bepridil or the IS (Table I). Therefore, the present method is an improvement of previous methods since the bepridil and IS HPLC peaks are not affected by medicines commonly co-administered with bepridil.

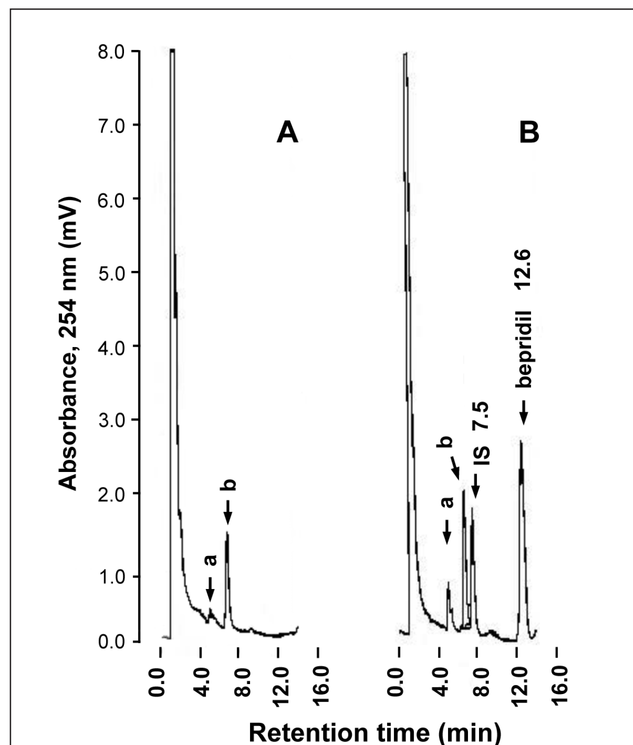

Figure 2. Representative chromatograms of blank plasma (A) and plasma spiked with 250 ng/mL of bepridil and 2.5 µg/mL IS (1-naphthol) (B). Peaks (a) and (b) originate from plasma. The retention times of bepridil and the IS are 12.6 and 7.5 min, respectively.

Linearity and goodness of fit

The peak height ratio (for each concentration of bepridil/IS) for a 5-point calibration curve was linear from 25 to 1000 ng/mL. The range of accuracy and precision of the back-calculated concentrations of the standard curve points was from 94.0% to 107.4% and 1.1% to 4.9%, respectively (Table II). The coefficient of correlation was consistently greater than 0.99 during the course of validation (data not shown).

Sensitivity

The lower LOD and LOQ were respectively 5 and 10 ng/mL for bepridil based on the S/N ratio of less than 3.0 and 8.0. These values were calculated based on the standard deviation of the baseline noise. Between-run accuracy and precision for the drug at LOQ were 95.1% and 3.2%, respectively. Our method showed a similar detection limit of 10 ng/mL in 500 µL human plasma with those of previous methods, one using a morphine analog of bepridil, available from a medical corporation, as the IS (4) and another using no IS (5). Our method could also measure a high concentration, 1000 ng/mL, which is practical in this study.

Accuracy and precision

Within-day accuracy ranged from 98.0% to 101.4% and between-day accuracy ranged from 95.6% to 104.4% (Table III). Precision ranged from 1.1% to 4.0% during between-day runs throughout the study. These ranges were considered acceptable. Accuracy and precision could be improved by increasing the number of extraction times. In comparison, previous method (4) gave a precision and accuracy of 2.0–6.3% and 1.0–2.6% as absolute values, respectively.

Table II. Result of Between-day Calibration Curves of Bepridil (*n* = 6)

Nominal conc. (ng/mL)	Measured conc. (ng/mL, mean + SD)	Precision (CV%)	Accuracy (%)
25	25.8 ± 1.1	4.4	103.0
100	99.4 ± 4.6	4.6	99.4
250	244.2 ± 2.8	1.2	97.7
500	499.6 ± 2.6	0.5	99.9
1000	998.5 ± 7.0	0.7	99.8

Table III. Results of Quality Control Samples Obtained During Within-day and Between-day Validation

QC sample	Low	Medium	High
Nominal conc. (ng/mL)	25	250	1000
Within-day (*n* = 9)			
Mean ± SD (ng/mL)	24.7 ± 1.2	253.9 ± 11.6	979.7 ± 49.4
Precision (CV%)	5.0	4.6	5.0
Accuracy (%)	98.9	101.4	98.0
Between-day (*n* = 4)			
Mean ± SD (ng/mL)	25.2 ± 0.8	250.8 ± 2.8	961.5 ± 38.2
Precision (CV%)	3.1	1.1	4.0
Accuracy (%)	100.8	100.3	96.1

Extraction recovery

Recovery of bepridil from the low, medium, and high QC samples was 104.4 ± 3.5%, 109.9 ± 1.1% and 110.8 ± 1.0%, respectively. Recovery of the IS was good, within the range of 22.9 ± 0.8% to 24.8 ± 0.2%. The extraction recovery of 1-naphthol used as the IS was low, because 1-naphthol is a very weakly acidic compound. During the extraction step, pH of the mixture was adjusted to 7.4 for improved extraction recovery of bepridil. 1-Naphthol was chosen as the IS, because it resulted in the best extraction recovery under the extraction conditions in this study, compared with 1-hydroxy-2-naphthoic acid, ethyl *p*-aminobenzoate and butyl *p*-aminobenzoate. Ng et al. (4) achieved a recovery of bepridil from plasma of 51.6%, by agitating the sample containing bepridil for 10 min on a table-top shaker with oscillation during extraction. We further improved recovery by performing the extraction step twice.

Stability of analytical solutions

Good stability of bepridil was observed in plasma at low (25 ng/mL), medium (250 ng/mL) and high (1000 ng/mL) concentrations at –20°C for 21 days (Figure 3). At low concentration, bepridil decreased from 100 to 82.6% after 42 days. Clusters formed in the plasma, and several peaks of unknown origin appeared on HPLC following long-term storage at –20°C. Such clustering may have affected the estimated stability at low concentrations of bepridil in the plasma samples.

Samples from patients

The concentration of bepridil in plasma samples from five patients was measured with AF. Blood samples were collected just before each dosage of bepridil. Patients displayed electrocardiogram sinus rhythms. The trough concentration of bepridil, referring to the lowest level of bepridil in the plasma, ranged from 233.9 to 347.4 ng/mL (Table IV), which was compatible with previous reported values. Benet (8) reported bepridil trough concentrations of 456 ± 326 ng/mL in 23 to 28 patients dosed with 200 mg bepridil twice daily; however, details regarding the patients and dosing intervals are unavailable. Taguchi et al. (5) measured the bepridil concentration in plasma from 34 Japanese

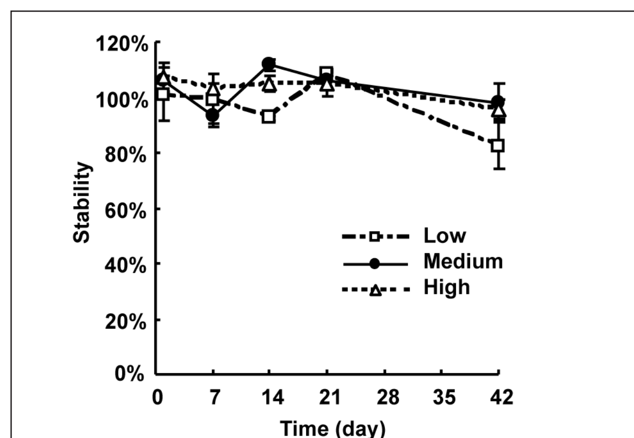

Figure 3. Result of the bepridil stability study at –20°C (*n* = 3). QC samples of low (□), medium (?) and high (△) concentrations of bepridil were prepared at 25, 250, and 1000 ng/mL, respectively.

Table IV. Pathological Data and Trough Concentration of Bepridil in Plasma of 5 Patients with Atrial Fibrillation

Patient no.	Age (years)	Gender (M/F)	Weight (kg)	Dose (mg/day)	Measured conc. (ng/mL, mean ± SD)	Precision (CV%)
1	85	F	51.0	100	312.7 ± 2.0	0.6
2	61	M	52.9	200	309 ± 1.8	0.6
3	69	F	57.0	100	327.7 ± 15.9	4.9
4	65	F	46.2	200	300.4 ± 4.9	1.6
5	84	F	68.4	100	233.5 ± 3.7	1.6

patients with arrhythmias; the concentrations ranged from 100 to 1500 ng/mL at 2–24 h after dosing with 100–200 mg daily. Overall, studies have used varying methods to evaluate bepridil, including investigation of the plasma concentration of bepridil at various times after dosing (5), as well as pharmacokinetics in healthy volunteers after single dosing (9), in patients with end-stage renal dysfunction (10), and in patients not suffering from cardiovascular disease (5,8).

Conclusion

In this study, we developed a method for quantifying bepridil hydrochloride in human plasma using easily obtainable materials. 1-Naphthol was used as the IS to measure the concentration of bepridil. In previous HPLC studies, Ng et al. (4) used as the IS a morphine analog of bepridil offered by a medical corporation, and Taguchi et al. (5) measured bepridil without an IS. Other methods for measuring bepridil plasma concentration have also been reported [i.e., gas chromatography (7) and reversed-phase thin-layer chromatography (6)]. However, the former method has limited application for routine clinical use, and the latter method is limited in quantitation and sensitivity compared with HPLC. Overall, our modified HPLC method achieves improved accuracy, precision and extraction recovery of bepridil compared with previous methods (4–6), and there is clearly no interference from other drugs used to treat AF. Using our method, future studies will investigate the relationship between bepridil hydrochloride in human plasma and the efficacy and occurrence of adverse effects in a larger number of patients with AF. The notable points of the present investigation are (1) the subjects were patients treated with bepridil for AF, (2) their therapeutic condition after dosing was clear, and (3) sampling points were fixed as troughs (Cmin).

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Manuscript received January 13, 2010;
revision received July 12, 2010.