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DETERMINATION OF BEPRIDIL IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, selective and sensitive assay has been developed for the determination of the anti-anginal drug, bepridil, in biological samples. The lowest concentration of bepridil which can be measured accurately and precisely in a 2-ml plasma or urine sample is 10 ng/ml. The standard curve is linear in the concentration range 10—2000 ng/ml. Accuracy and precision of the assay, expressed as relative deviation and coefficient of variation (inter-run) are < 6.5% at all concentrations in the linear range. No interfering peaks are observed. Using an automatic injector and a laboratory computer system, 48 samples are analyzed routinely in an 8-h day.

INTRODUCTION

Bepridil hydrochloride, β-[(2-methylpropoxy)methyl]-N-phenyl-N-(phenylmethyl)-1-pyrrolidineethanamine monohydrochloride monohydrate, is a new cardiovascular drug currently undergoing clinical evaluation for the treatment of angina pectoris. Its anti-anginal and anti-arrhythmic properties have been demonstrated in animal studies [1—7] and in clinical studies [8, 9].

Recently a gas chromatographic (GC) assay for bepridil was reported [10] which uses nitrogen-specific detection. This GC assay has a detection limit of 5 ng/ml using 2—3 ml of plasma. However, some clinical plasma samples may require reanalysis with adjusted amounts of added internal standard because they fall outside the anticipated concentration range. A gas chromatographic—mass spectrometric (GC—MS) assay for the determination of both stable-isotope-labelled and unlabelled bepridil in human plasma was also reported [11]. Accuracy and precision for the GC—MS assay were only 30% at 20 ng/ml or below.

In the present paper, a new high-performance liquid chromatographic

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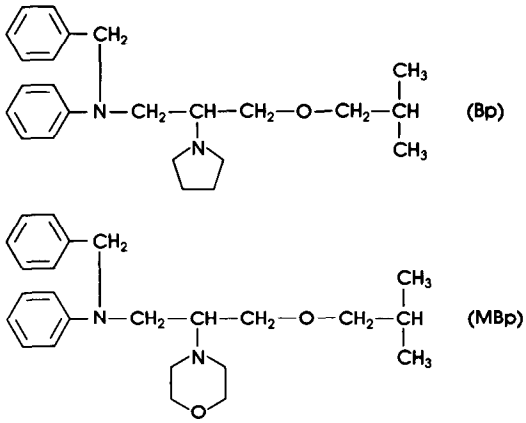

Fig. 1. Chemical structures of bepridil (Bp) and the morpholine analogue of bepridil (MBp).

(HPLC) assay for the determination of bepridil in biological fluids is described. The detection limit is 10 ng/ml. The standard curve is linear between 10 and 2000 ng/ml. The method is highly reproducible (inter-run coefficient of variation $< 6.5\%$) and accurate (relative deviation $< 3\%$). The assay has been automated to analyze 48 or more samples during an 8-h working day.

EXPERIMENTAL

Reagents

Hexane and methanol were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Ammonium hydroxide (58%) and orthophosphoric acid (85%) were of ACS grade (Mallinckrodt, Paris, KY, U.S.A.). Monobasic potassium phosphate and dibasic sodium phosphate used in the preparation of the pH 7.4 buffer were of certified ACS grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The 10% triethylamine (TEA) solution used for the mobile phase was prepared by diluting 100 ml of 99% TEA (Eastman Kodak, Rochester, NY, U.S.A.) to 1 l with distilled water followed by titration to pH 3.3 with orthophosphoric acid (85%).

A morpholine analogue of bepridil (Fig. 1, MBp) was used as the internal standard. Bepridil (Fig. 1, Bp) was obtained as the hydrochloride salt (McNeil Pharmaceutical, Spring House, PA, U.S.A.).

Liquid chromatography

The HPLC system consisted of a Beckman Model 112 solvent delivery system and a Beckman Model 160 UV absorbance detector equipped with a 254-nm wavelength filter. The column was 10 cm × 4.6 mm I.D. packed with 5-μm RP-18, Spheri-5 sorbent (Brownlee Labs., Santa Clara, CA, U.S.A.). A 3 cm × 4.6 mm I.D. 10-μm RP-18 guard column from Brownlee Labs. was also used. The mobile phase was methanol—water—10% TEA solution (68:22:10, v/v). The mobile phase was prepared fresh daily and filtered through a 0.45-μm Millipore® filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approximately 30 ml of the mobile phase prior to use. After conditioning the column, the flow-rate was maintained at 2.0 ml/min. The

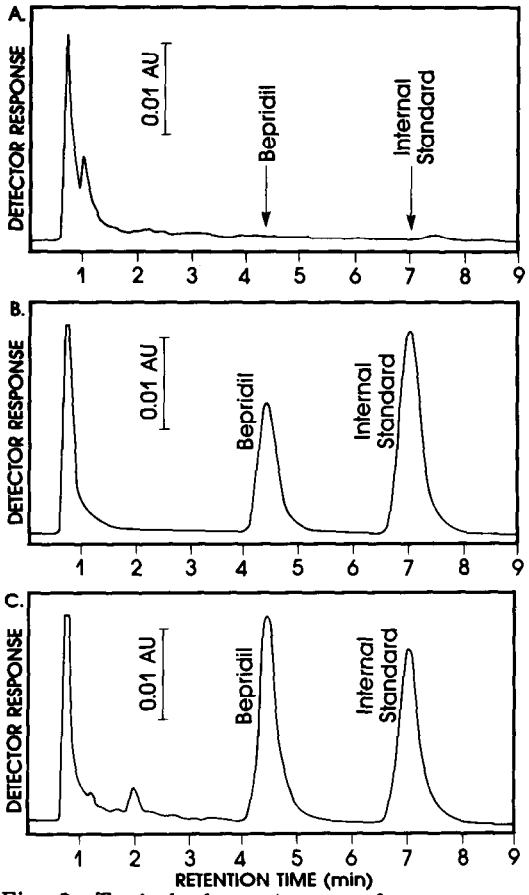

Fig. 2. Typical chromatograms from extracted human plasma samples. (A) Blank. (B) Sample seeded with 400 ng/ml of bepridil and 800 ng/ml of the internal standard. (C) A clinical sample obtained 2 h following a 400-mg oral dose; the bepridil concentration was calculated to be 719 ng/ml.

retention times were 4.4 min for bepridil and 7.0 min for the internal standard (Fig. 2). Samples were injected using a Waters Intelligent Sample Processor (WISP-710B, Waters Assoc., Milford, MA, U.S.A.).

Equipment

Disposable screw-top bottles (volume, 14.5 ml) with polyethylene-lined caps and 12-ml centrifuge tubes (conical bottom) were used for extraction. Prior to use, all glassware was soaked in detergent for 2 h, rinsed thoroughly with distilled water and heat-treated for 3 h at 270°C. Polyethylene-lined screw caps were soaked in *n*-heptane for 1 h and dried at 60°C before use.

Plasma standard solutions

Plasma standards (volume, 10.0 ml) containing 10—1000 ng of bepridil per ml of plasma were prepared as follows: 0.5 ml of a methanolic solution of bepridil hydrochloride (conversion factor to bepridil free base = 1.149), containing the appropriate amount (200—20,000 ng equivalent) of the bepridil free base, was added to 9.5 ml of drug-free plasma.

Extraction procedure

An aliquot of plasma or urine (1.0—2.0 ml), containing bepridil as a standard or an unknown was placed in a 14.5-ml disposable screw-top bottle. To this were added 2.0 ml of pH 7.4 phosphate buffer, 0.4 ml of a methanolic internal standard solution containing 1600 ng of internal standard (MBp) and 9.5 ml of hexane. The capped bottle was shaken for 10 min on a table-top shaker (Eberbach) at 240 oscillations per minute and centrifuged at 1000 *g* for 10 min. An 8-ml aliquot of the supernatant hexane layer was pipetted into another 14.5-ml screw-top bottle containing 4.0 ml of methanol and 0.2 ml of 0.58% ammonium hydroxide solution. The bottle was capped, shaken and centrifuged as before. A 7.0-ml aliquot of the supernatant hexane layer was transferred to a 12-ml centrifuge tube and evaporated to dryness under a stream of dry nitrogen at room temperature. The residue was reconstituted in 200 μl of the HPLC mobile phase. This was mixed on a Vortex Genie® (Scientific Instruments, Springfield, MA, U.S.A.) at a speed setting of 6 for 10 sec. All of the resulting solution was transferred to a limited-volume insert tube (Waters Assoc.) which was placed onto the sample carousel of the Waters Intelligent Sample Processor (WISP-710B). A 100-μl aliquot of this solution was subsequently injected into the HPLC system.

Quantitation and data handling

Standard curve data were generated by analyzing a series of plasma standards (10—2000 ng/ml). Data were analyzed by linear-regression analysis (peak height ratios versus plasma concentration) using the reciprocal of the variance of the peak height ratios as the weighting factor. Concentrations of bepridil in unknown plasma samples were determined using the calculated peak height ratios and the linear-regression equation.

A Hewlett-Packard 3354 Lab Automation System was used for automatic data acquisition, temporary data storage, data analysis and report generation. Calibration functions, calculated bepridil concentrations and final reports were generated using internally developed application software.

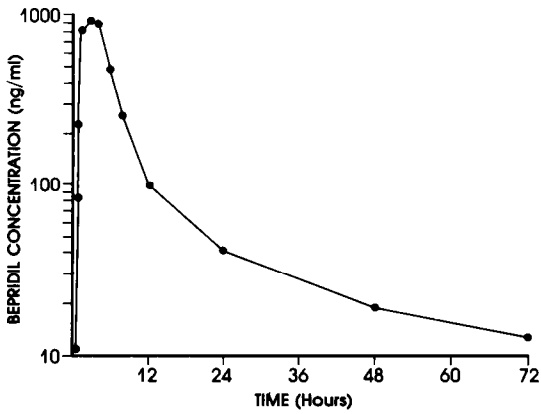

Fig. 3. Bepridil plasma concentration versus time following oral administration of a single 400-mg dose of bepridil hydrochloride to a fasted healthy human volunteer.

RESULTS AND DISCUSSION

Sensitivity

Bepridil and the internal standard (MBp) absorb ultraviolet strongly at 254 nm in the acidic mobile phase (for bepridil $\epsilon_{254\,nm}$ = 12,500). When 1 ng of bepridil was injected into the liquid chromatograph under the above conditions, a peak with a signal-to-noise ratio of 33 was obtained. The lowest concentration of bepridil that has been determined quantitatively in 2-ml plasma/urine samples is 10 ng/ml (relative standard deviation $\leqslant$ 6.5% for precision). This detection limit is adequate for therapeutic drug monitoring since the average plasma concentration of bepridil at 72 h following oral administration of a single therapeutic dose (400 mg) is approximately 15 ng/ml (Fig. 3).

Stability

Freshly prepared plasma/urine standard solutions were compared to plasma/urine standard solutions frozen at —5°C for one month. The variations in peak height ratios at each drug concentration between 10 and 2000 ng/ml were insignificant. Furthermore, bepridil and the internal standard were found to be stable in dried plasma/urine extract or in mobile phase at room temperature overnight. Therefore, injection of samples can be performed on the day following extraction without observable changes in peak height ratios.

Recovery

Recovery of bepridil from plasma/urine was calculated by comparing the slope of the detector response curve to that of the standard curve (peak height for internal standard normalized to 1.0). This ratio was found to be 51.6 ± 0.2%. After correction for volume losses, the intrinsic extraction efficiency for bepridil from plasma/urine was determined to be 66.3 ± 0.3%. The extraction efficiency for the internal standard (at 800 ng/ml) was 54.2 ± 2.6% (mean ± S.D. from twelve determinations).

Selectivity

The selectivityof the assay is shown in Fig. 2. No interfering peaks due to endogenous materials or metabolites of bepridil were observed in plasma/urine samples from various studies involving mouse, rat, rabbit, dog, monkey and man. The extraction procedure is highly selective for bepridil. An experiment was conducted in which plasma samples from eight rats were pooled. These samples were obtained 3 h following oral administration of a 100 mg/kg dose of [^{14}C]bepridil. Samples from this pool were assayed for total radioactivity and extracted using the present procedure.

Total radioactivity and unchanged bepridil content in the resultant plasma extracts were determined. The results, when expressed as percentages of the total radioactivity in the samples before extraction, were 5.1 ± 0.2% and 3.2 ± 0.2%, respectively (six determinations each). Because the extraction efficiency for bepridil from plasma is 66%, it can be calculated that the overall extraction efficiency for bepridil metabolites is only 2.0 ± 0.2%. The 33-fold difference in selectivity is attributed to the hexane—methanol partitioning step.

TABLE I

SUMMARY OF STANDARD CURVE DATA GENERATED ON THREE CONSECUTIVE DAYS OF ANALYSIS OF BEPRIDIL IN PLASMA (n = 6)

Actual concentration (ng/ml)	Calculated concentration* (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
10	10.0	0.2	2.4	0.0
25	25.0	1.3	5.2	0.0
50	49.1	3.1	6.3	−1.8
100	101.2	5.2	5.1	1.2
200	204.4	11.2	5.5	2.2
400	396.2	7.9	2.0	−1.0
600	611.0	27.5	4.5	1.8
800	793.3	30.9	3.9	−0.8
1000	1025.9	43.1	4.2	2.6

*Calculated from the equation: $[\text{Bepridil}] = \frac{(\text{peak height ratio} - 0.004)}{2.35 \cdot 10^{-3}}$ where $2.35 \cdot 10^{-3}$ and 0.004 are the slope and intercept of the regression equation, respectively. The regression equation was obtained by method of least squares with data weighted by 1/variance.

Standard curve

Standard curve data generated by analyzing plasma standard solutions are presented in Table I. Linear-regression analysis (peak height ratios versus bepridil plasma concentrations) using reciprocals of variance as weighting factors gave a slope of $2.35 \cdot 10^{-3} \pm 0.01 \cdot 10^{-3}$ (mean ± S.D.), a y-intercept of 0.004 ± 0.003 and a correlation coefficient of 0.999 with a Student's t of 171.

Similar standard curve data have also been generated for spiked urine samples. Excellent accuracy and precision were obtained. For smaller sample volumes, assay sensitivity was reduced proportionally. Using 1-ml plasma samples, the standard curve was found to be linear between 20 and 4000 ng/ml.

TABLE II

DATA SUMMARY OF SEEDED CONTROL SAMPLES TO ILLUSTRATE THE LONG-TERM STABILITY IN INTER-RUN ASSAY PRECISION (n = 36)

Actual bepridil concentration (ng/ml)	Calculated bepridil concentration* (ng/ml)	Inter-run assay precision** (%)
100	98.8	6.4
400	394.4	5.6
800	805.4	5.3

*Mean of 36 values derived from 18 daily calibration curves.
**Relative standard deviation.

Accuracy and precision

Accuracy and precision of the assay were measured by the relative difference between the mean experimental bepridil concentration and the theoretical value, and the relative standard deviation, respectively (Table I). Duplicate frozen seeded control samples at three different concentrations were blind-coded and analyzed with plasma samples from each clinical study. The data for one such study are presented in Table II (inter-run precision $\leqslant$ 7% at all three concentrations over a period of one month).

Application of the procedure to plasma samples

To date, the procedure has been employed successfully in analyzing over 5000 biological samples from mouse, rat, rabbit, dog, monkey and man. Fig. 3 shows a typical plasma concentration versus time profile from a subject following oral administration of a single 400-mg capsule dose of bepridil hydrochloride. Serial blood samples were drawn up to 72 h after dosing. The 13 ng/ml plasma concentration observed at the final time point is above the detection limit of the assay (10 ng/ml).

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REFERENCES

1 D. Cosnier, P. Duchene-Marullaz, G. Rispat and G. Streichenberger, Arch. Int. Pharmacodyn. Ther., 225 (1977) 133.
2 M.T. Michelin, M. Cheucle and P. Duchene-Marullaz, Therapie, 32 (1977) 485.
3 P. Piris, M. Beaughard, D. Cosnier and C. Labrid, Arch. Int. Pharmacodyn. Ther., 235 (1978) 147.
4 C. Chassaing, N. Moins, J. Lavarenne and P. Duchene-Marullaz, J. Pharmacol., 8 (1977) 503.
5 M. Boucher and P. Duchene-Marullaz, Arch. Int. Pharmacodyn. Ther., 233 (1978) 65.
6 D.R. Harder and N. Sperelakis, J. Cardiovasc. Pharmacol., 3 (1981) 906.
7 N.F. Reifart, M. Zierler, A. Taylor and S. Khuri, Clin. Res., 30 (1982) 215A.
8 J.C. Canicave, J. Deu and F.X. Lesbre, Lyon Med., 243 (1980) 107.
9 D. Granatelli, D. Germa and L. Tatibouet, Quest. Med., 15 (1981) 1041.
10 J. Vink, H.J.M. van Hal, J.-F. Pognat and J.-L. Bouquet Des Chaux, J. Chromatogr., 272 (1983) 87.
11 J. Vink, H.J.M. van Hal, F.M. Kaspersen and H.P. Wijnand, Int. J. Mass Spectrom. Ion Phys., 48 (1983) 217.