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DETERMINATION OF THE ANTI-ISCHAEMIC DRUG BEPRIDIL IN HUMAN PLASMA USING GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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SUMMARY

An assay has been developed to determine the anti-ischaemic drug bepridil (as its free base) in human plasma. The assay procedure comprises *n*-hexane extraction from basic plasma and gas chromatography using nitrogen-selective detection. An analogue of bepridil is used as internal standard. The accuracy and precision of the assay is determined by repeated analyses of drug-free plasma samples spiked with 5, 10, 20, 100, 400 and 1000 ng of bepridil per ml of plasma. The accuracy, defined as the relative difference between the mean bepridil concentration found and the true value, was 8% or better. The precision (relative standard deviation) was 13% at the 5 ng/ml level and 5% at the 1000 ng/ml level. The assay is suitable to monitor routinely bepridil plasma levels during clinical studies.

INTRODUCTION

In animal studies, bepridil (β-[(2-methylpropoxy)methyl]-N-phenyl-N-phenylmethyl-1-pyrrolidineethanamine; Fig. 1, 1) has been shown to possess anti-anginal [1 -3] and anti-arrhythmic [4, 5] properties. In a recent clinical study [6], the efficacy of bepridil in the treatment of angina pectoris has been demonstrated.

To assess the pharmacokinetics of bepridil and the bioavailability of bepridil from different pharmaceutical formulations or to measure plasma levels of bepridil in clinical efficacy studies, assay methods have been developed for its

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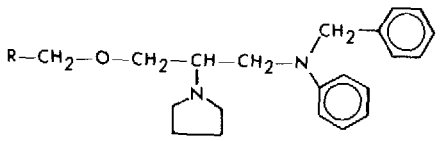

Bepridil 1: R = $(CH_3)_2CH$

Internal standard 2: R = H

Fig. 1. Structure of bepridil and internal standard.

quantitative determination. In the present paper, the assay for the determination of bepridil in human plasma is reported. The assay is based on gas chromatography (GC) with nitrogen-selective detection using an analogue of bepridil as internal standard. Gas chromatography with nitrogen detection has been established as a reliable method for the quantitation of nitrogen-containing drugs in biological fluids using closely related compounds (in structure) as internal standards [7].

MATERIALS AND METHODS

Chemicals and glassware

The internal standard (Fig. 1, 2), the methyl-ether analogue of bepridil, was obtained from CERM-Reti, Riom, France.

The solvents *n*-hexane and methanol (Merck, Darmstadt, G.F.R.) were of Uvasol and analytical-reagent grade, respectively. Disodium hydrogen phosphate was purchased from Merck.

Solvent extraction of plasma aliquots was performed in 20-ml screw-capped disposable glass vials. The 10-ml conical glass tubes and 250-μl screw-capped conical glass vials (used for extract concentration and injection into the gas chromatograph, respectively) were rinsed three times with ethanol prior to use. In experiments to determine the recovery of bepridil from plasma during extraction and concentration, tritium-labelled bepridil was used. [^{3}H]Bepridil was obtained from the Organic Synthesis Group, Drug Metabolism R&D Labs, Organon International B.V., Oss, The Netherlands.

Gas chromatography

A Hewlett-Packard gas chromatograph Model 5710A, equipped with a 4 m × 2 mm I.D. glass column packed with 3% OV-17 on Gas-Chrom Q 80—100 mesh, was used. Chromatographic analyses were performed isothermally at a column temperature of 270°C. The temperature of the injector and N/P detector (Hewlett-Packard Model 18789A) was 300°C. Nitrogen was used as carrier gas at a flow-rate of 30 ml/min. The gas flow-rate for the N/P detector was 3 ml/min for hydrogen and 50 ml/min for air. The collector voltage was 15—20 V.

Assay procedure

The design of the clinical study, the administered dose and time of blood sampling was used to predict the bepridil plasma level which in turn was used

as the rationale to determine the volume of plasma to be processed. For expected bepridil levels in the range of 10—100 ng/ml, an aliquot of 1 ml of plasma was taken; for expected bepridil levels below 10 ng/ml generally more than 1 ml (maximum 3 ml) of plasma was processed; at expected bepridil levels above 100 ng/ml usually less than 1 ml of plasma was taken with a minimum manageable volume of 100 μl. The plasma aliquots were pipetted into 20-ml disposable glass vials. The internal standard was added to plasma as an aqueous solution containing 100 or 1000 ng/ml. The amount of internal standard added was approximately the same as the anticipated amount of bepridil in the plasma sample. The plasma was mixed thoroughly by vortexing and allowed to equilibrate for at least 30 min. The plasma pH was adjusted to approximately 8.9 by addition of a saturated Na_2HPO_4 solution. The volume of the Na_2HPO_4 buffer equalled the volume of plasma taken, with a minimum of 0.5 ml. The diluted, basic plasma was mixed thoroughly. The plasma sample was extracted with approximately 10 ml of *n*-hexane by vortexing, followed by centrifugation at 1500 *g*. The *n*-hexane extract was transferred to a 10-ml conical glass tube and the solvent evaporated under a gentle stream of nitrogen at 40°C. A second extraction of the water phase was carried out with approximately 8 ml of *n*-hexane. The organic layer was evaporated to dryness under nitrogen in the 10-ml tube containing the residue obtained after the first *n*-hexane extraction. The wall of the glass tube was rinsed thoroughly with 500 μl of methanol, which was evaporated under nitrogen at 40°C. The residue was taken up in 200 μl of methanol and transferred to a 250-μl conical glass tube. The extract was concentrated under a gentle stream of nitrogen at 40°C and taken up in 20—100 μl of methanol. An aliquot of this methanolic solution was injected into the gas chromatograph equipped with a nitrogen-sensitive detector.

Calibration

A series of drug-free plasma samples spiked with bepridil and its internal standard in different concentration ratios were prepared for calibration purposes. The series comprised plasma samples with a 1:1 concentration ratio of bepridil to internal standard and with concentration ratios around this unity ratio, e.g. 1:3, 1:2, 2:1 and 3:1. In each sample prepared for calibration the amount of bepridil and internal standard did not differ substantially from the amounts expected in the clinical samples. The samples prepared for calibration were processed in the same way as the clinical plasma samples received for analysis.

Quality control on the results of analysis

On each day of measurement, clinical plasma samples containing bepridil, samples prepared for calibration, drug-free plasma samples (blanks) and drug-free samples spiked with known amounts of bepridil were analyzed randomly. Analysis of drug-free plasma samples with and without bepridil added, ensured continuous quality control of the assay procedure. Clinical plasma samples in which the ratio of bepridil to internal standard eventually proved to be outside the upper or lower limit used in the series of calibration samples, were re-analyzed with adapted amounts of internal standard.

Automation and data handling

The laboratories involved in the development of an assay for bepridil had access to different facilities for automation and data handling. At Organon the following instruments were employed to facilitate the routine determination of bepridil plasma levels: a programmable microprocessor-controlled handpipette (MicroLab P from Hamilton, Bonaduz, Switzerland) was used to take aliquots of the plasma samples and to add the internal standard to plasma; a Hewlett-Packard 7672A automatic sampler was used for injection of plasma extracts into the GC column.

A Varian CDS 400 V laboratory data system equipped with a 983 K disc was used for data acquisition, subsequent data integration and temporary data storage. The integrated data were transmitted as peak heights found within a specified retention time window to a DEC PDP-11/RSTS computer network. Calibration functions and bepridil plasma levels were calculated using application programs written in BASIC.

At CERM-Reti, the gas chromatograph was equipped with a Hewlett-Packard 7672A Automatic Sampler. A Hewlett-Packard integrator type 3390A was used for signal integration. Further data handling was carried out with a Texas Instruments TI 51-III calculator using internally developed application programs.

Clinical study

To demonstrate the applicability of the assay method, plasma samples from a clinical study were analyzed. The dosage regimen in this study was such that steady-state levels were reached within two days. Bepridil monohydrochloride monohydrate was administered as an intravenous loading dose of 2 mg/kg given as an infusion over a period of 5 min. Four, 12 and 20 h after the infusion, oral doses of three tablets containing 100 mg of bepridil monohydrochloride monohydrate each were given; this was followed by oral doses of one 100-mg tablet at intervals of 8 h.

Blood samples were collected at regular intervals after the end of infusion and during oral drug administration just prior to each new dose. The blood was centrifuged and plasma samples were stored at −20°C until required for analysis.

RESULTS AND DISCUSSION

Recovery of bepridil and internal standard from plasma

In triplicate experiments with labelled bepridil at a plasma level of 100 ng/ml, 68.1% (S.D. 0.7%) of the amount of bepridil added to drug-free plasma appeared in the methanolic solution, from which aliquots were subjected to GC analysis. The observed "loss" of 32% of bepridil during the assay procedure included extraction as well as manipulation losses. However, this acceptable recovery of bepridil in the methanolic solution did not guarantee a similar recovery of the analogue used as internal standard. It was observed during the initial phase of the development of the assay that, using plasma samples from different sources spiked with known amounts of bepridil and internal standard, the peak height ratios of bepridil to internal standard fluctuated considerably. This phenomenon was attributed to differences between the plasma protein

binding of bepridil and its internal standard. Incubation of a bepridil-containing plasma sample with the internal standard for at least 30 min, dilution of plasma with the aqueous solution of the internal standard, addition of the concentrated Na_2HPO_4 buffer solution and the double *n*-hexane extraction were the measures taken to obtain reproducible peak height ratios of bepridil to internal standard.

Decomposition problems

Additional peaks not well separated from the peaks corresponding to bepridil and the internal standard appeared in the gas chromatograms when the GC column had been in use for a long time period. This was due to decomposition of bepridil and, to a lesser extent, of the internal standard. The decomposition problem was avoided by applying inert quartz wool to the injection side of the packed column and by regular changing of the quartz wool followed by careful conditioning of the column.

Selectivity

The selectivity of the assay is demonstrated in Fig. 2, which gives gas chromatograms of processed plasma samples from drug-free plasma, drug-free plasma spiked with a known amount of bepridil and human plasma from a clinical study. Interference from endogenous human plasma components was not observed at the retention times where bepridil and the internal standard eluted from the GC column. Interference from possible bepridil biotransformation products was unlikely judging from the gas chromatogram obtained after identical processing of a urine sample from a volunteer receiving chronic bepridil treatment. It is considered that the human metabolites of bepridil are either too polar to be extracted under the assay conditions used or can easily be separated from bepridil and its internal standard on the GC column.

Calibration curve

The calibration curve for the bepridil assay was calculated by linear or polynomial regression analysis. With the polynomial regression analysis using $y = ax^2 + bx + c$, where for example $a = 0.022$, $b = 0.692$, and $c = -0.021$, the deviation from linearity was, as expected, small.

Detection limit

From the signal and noise in the chromatograms obtained after processing a 3-ml plasma sample spiked with 3 ng of bepridil per ml (Fig. 2B), it was deduced that 1 ng of bepridil per ml of plasma could be detected with a signal-to-noise ratio of more than 10.

Accuracy and precision

The relative difference between the mean bepridil concentration found and the true value, and the relative standard deviation as measures for the accuracy and precision of the bepridil assay, respectively, were determined by analyzing drug-free plasma samples spiked with known amounts of bepridil. These spiked samples were analyzed concomitantly with plasma samples received from the clinical study over a period of one month. The accuracy and precision at the

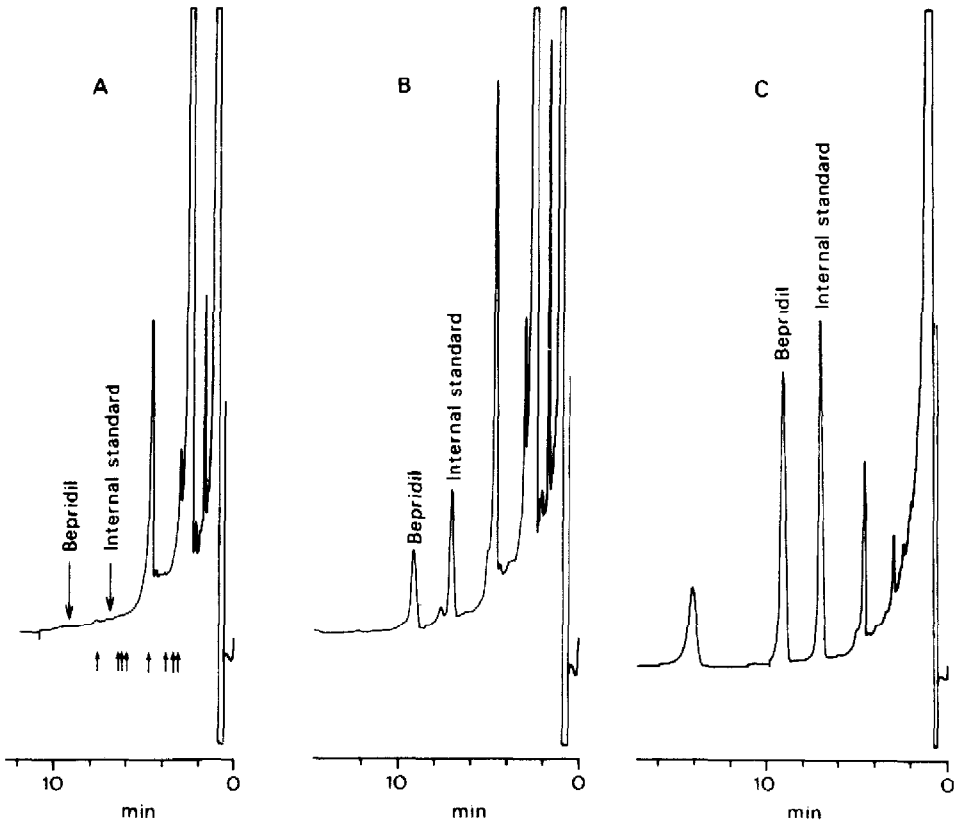

Fig. 2. Gas chromatograms of processed human plasma. (A) Drug-free human plasma. The expected positions of bepridil, the internal standard and metabolites extractable from human urine are indicated by arrows. The volume of plasma processed was 1 ml. (B) Spiked drug-free human plasma. Three nanograms of bepridil and 3 ng of internal standard per ml were added to 3 ml of blank plasma. (C) Clinical plasma sample. Fifty nanograms of the internal standard were added to 0.5 ml of the sample; the bepridil concentration was calculated to be 140 ng/ml.

5, 10, 20, 100, 400, 1000 ng/ml plasma levels are given in Table I.

The standard deviation as a function of the concentration can also be expressed in terms of an error model used for data point weighting in pharmacokinetic curve-fitting programs [8]. According to this error model, $s^2 = a(\bar{C})^b$, where s^2 is the square of standard deviation (variance), a and b are the appropriate coefficients and $\bar{C}$ is the mean concentration. A plot of the log (variance) versus the log (mean concentration) yielded a straight line ($r = 0.997$). The error model for the bepridil assay was $s^2 = 0.0186(\bar{C})^{1.731}$, which could be used to estimate the standard deviation at different plasma levels. For instance, at the 250 ng/ml level, the relative standard deviation as a measure of precision is expected to be 6.5%.

Routine application of the assay

The time course of plasma levels in one patient from the clinical study is shown in Fig. 3. The bepridil (free base) plasma level was 8 μg/ml 2 min

TABLE I

ASSAY CHARACTERISTICS DETERMINED BY ANALYSIS OF 1-ml DRUG-FREE PLASMA SAMPLES SPIKED WITH DIFFERENT AMOUNTS OF BEPRIDIL

Bepridil added (ng/ml)	No. of determinations	Mean value found (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
1000	15	996	51	5	−1
400	20	392	24	6	−2
100	21	95.2	8.4	9	−5
20	16	20.2	1.7	8	+1
10	5	10.2	0.8	8	+2
5	5	4.6	0.6	13	−8

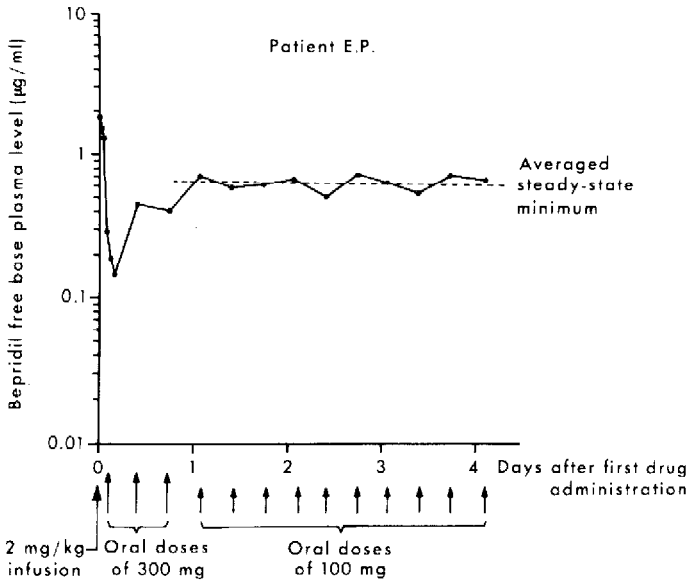

Fig. 3. Bepridil plasma levels vs. time in a clinical experiment undertaken by patients receiving an intravenous loading dose followed by oral drug administrations.

after the infusion finished; the plasma level declined to 140 ng/ml in the 4-h period preceding the first oral administration of 300 mg of bepridil monohydrochloride monohydrate, while the steady-state plateau with an average plasma level of 630 ng/ml was reached in approximately one day.

CONCLUSIONS

The GC assay method developed for the determination of bepridil in human plasma is sensitive, selective and applicable for routine measurement of plasma levels of bepridil in clinical studies. The use of GC with nitrogen-sensitive detection has produced an assay simply involving extraction of bepridil and the internal standard from basic plasma, followed by chromatography and detec-

tion without the need for purification of plasma extracts prior to chromatography. The selectivity for the determination of the unchanged drug with regard to possible metabolites and endogenous plasma components was guaranteed by the conditions chosen for extraction, GC separation and detection. The assay characteristics, expressed in terms of the precision and accuracy determined by analyzing spiked human plasma samples, allow bepridil plasma level measurement in pharmacokinetic and bioavailability studies.

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REFERENCES

1 M.T. Michelin, M. Cheucle and P. Duchene-Marullaz, Therapie, 32 (1977) 485.
2 D. Cosnier, P. Duchene-Marullaz, G. Rispat and G. Streichenberger, Arch. Int. Pharmacodyn. Ther., 225 (1977) 133.
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 J.C. Canicave, J. Deu and F.X. Lesbre, Lyon Med., 243 (1980) 107.
7 A. Richens and V. Marks, Therapeutic Drug Monitoring, Churchill Livingstone, Edinburgh, 1981.
8 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publications, Hamilton, IL, 1975, p. 288.