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LIQUID CHROMATOGRAPHIC DETERMINATION OF PINACIDIL, A NEW ANTIHYPERTENSIVE DRUG, AND ITS MAJOR METABOLITE, PINACIDIL N-OXIDE, IN PLASMA

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SUMMARY

Two procedures are described, one for the determination of pinacidil, the other for the determination of both pinacidil and its metabolite, pinacidil N-oxide, in plasma When only parent drug levels are required, the plasma proteins are precipitated with acetonitrile, the solids discarded and the supernatant is evaporated to dryness The residue is then reconstituted for analysis For the determination of both drug and metabolite, the analytes are selectively retained from plasma on a solid-phase extraction column and eluted with methanol After evaporation to dryness, the residue is reconstituted in mobile phase Both procedures utilize reversed-phase liquid chromatographic separations with ultraviolet detection The limits of detection are 10 ng/ml pinacidil in plasma and 5 ng/ml each of pinacidil and pinacidil N-oxide in plasma for the two procedures, respectively

INTRODUCTION

Pinacidil (PINDAC™), (±)-N''-cyano-N-4-pyridinyl-N'-(1,2,2-trimethylpropyl)guanidine monohydrate (I, Fig. 1) is a new potent direct-acting vasodilator currently undergoing clinical trials as an antihypertensive agent [1–12] It is almost completely absorbed following oral administration [1] and is a significantly more potent antihypertensive agent than hydralazine both in man and in animals [2–5] Pinacidil has been shown to be effective in the treatment of hypertension as monotherapy and in combination with diuretics and β-blockers [6—9]

The principle metabolite of pinacidil, pinacidil N-oxide (II, Fig 1), possesses only about one fourth of the activity of the parent [10] It is unlikely to contribute to the therapeutic effect of pinacidil in patients with normal kidney

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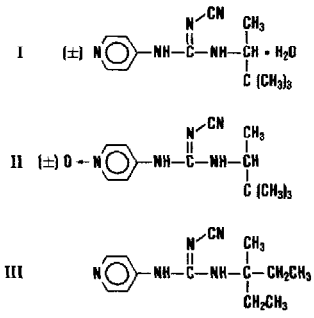

Fig 1 Structures of pinacidil (I), pinacidil N-oxide (II) and the internal standard (III) I = (±)-N''-cyano-N-4-pyridinyl-N'-(1,2,2-trimethylpropyl)guanidine monohydrate, II = (±)-N''-cyano-N-(1-oxido-4-pyridinyl)-N'-(1,2,2-trimethylpropyl)guanidine, III = N'-cyano-N-(1-ethyl-1-methylpropyl)-N'-4-pyridinylguanidine

function; however, renal impairment may result in elevated metabolite plasma concentrations.

To facilitate pharmacokinetic studies and therapeutic monitoring of pinacidil, a sensitive and selective method for the determination of drug and metabolite in plasma was needed. A previously reported method [1] employs a liquid- liquid extraction followed by high-performance liquid chromatographic analysis. This procedure is suitable for the determination of pinacidil in plasma but yields poor results for pinacidil N-oxide owing to the low extraction efficiency of this compound from aqueous media During the course of our work, two methods have been developed and successfully applied to the analysis of a large number of samples from animals and man. This report describes both procedures, one for use when only parent drug concentrations are to be determined and one which is selective for both pinacidil and pinacidil N-oxide.

EXPERIMENTAL

Chemicals and reagents

Pinacidil monohydrate, pinacidil N-oxide and the internal standard, N'-cyano-N-(1-ethyl-1-methylpropyl)-N'-4-pyridinylguanidine (Fig 1) were obtained from Eli Lilly (Indianapolis, IN, U S A) Sep-Pak® C_{18} cartridges were purchased from Waters Assoc (Milford, MA, U.S.A.). All solvents used were distilled-in-glass Morpholine (certified ACS grade) was obtained from Fisher Scientific (Fair Lawn, NJ, U S.A). All other chemicals were of analytical-reagent grade

Column liquid chromatography

The liquid chromatograph consisted of a Waters Model 6000A pump equipped with a Dupont Model 852 variable-wavelength detector (Dupont, Wilmington, DE, U.S.A.), an autoinjector (WISP®, Model 710B, Waters Assoc), and a Model LC-22 column temperature controller (Bioanalytical Systems, West Lafayette, IN, U S.A.). The column employed was stainless-steel, 25 cm × 4 6 mm I.D , packed with 6-μm Zorbax C_8 particles (Dupont) protected by a

guard column (Waters Assoc) packed with CO-Pell® ODS (Whatman, Clifton, NJ, U S.A). A Hewlett-Packard Model 1000 computer was used for on-line data acquisition and subsequent calculations.

Eluent system A (for determination of pinacidil alone) was prepared by mixing 450 ml of 0.01 *M* ammonium phosphate, pH 7 0, with 550 ml of methanol The eluent flow-rate was 1.5 ml/min and the column temperature 35°C Detection of pinacidil was made at 254 nm.

Eluent system B (for the determination of both pinacidil and pinacidil N-oxide) was prepared by mixing 550 ml of 0.1 *M* sodium acetate with 1.0 ml of morpholine The pH was adjusted to 4 0 with glacial acetic acid and 450 ml of methanol were added. The resulting solution was mixed well, filtered and degassed before use. The eluent flow-rate was 1 5 ml/min and the column temperature 40°C Pinacidil and pinacidil N-oxide were detected at 284 nm.

Standard solutions

Standards in plasma were prepared to contain 20, 50, 100 and 200 ng/ml pinacidil or pinacidil and pinacidil N-oxide, depending on whether drug or both drug and metabolite were to be determined The internal standard solution was prepared by dissolving 1 mg into 200 ml of 0 01 *M* hydrochloric acid.

Sample preparation procedures

Method A (for determination of pinacidil only) To 1 0 ml of plasma sample or standard, placed in a disposable centrifuge tube with a PTFE-lined screw cap, were added 50 μl of the internal standard solution and 2 ml of acetonitrile The tubes were capped and mixed by vortexing, then allowed to stand for 5 min. After a second vortexing, the tubes were centrifuged (500 *g*) for 5 min. The clear supernatant was transferred to a second tube and evaporated to dryness under nitrogen The residue was dissolved in 200 μl of methanol–water (50·50), and 100 μl were injected.

Method B (for determination of pinacidil and pinacidil N-oxide) To 1.0 ml of plasma sample or standard, placed in a disposable centrifuge tube with a PTFE-lined screw cap, 50 μl of the internal standard solution were added and mixed by vortexing The mixture was transferred to a glass syringe to which a pre-conditioned Sep-Pak® C_{18} cartridge was attached The cartridge had been prepared by the application of 3 ml of methanol followed by 5 ml of distilled, deionized water The sample was pressed through the cartridge followed by 5 ml of distilled deionized water and 5 ml of a methanol–water (20.80) mixture. The analytes were eluted from the cartridge with 4 ml of methanol which was evaporated to dryness under nitrogen The residue was dissolved in 200 μl of mobile phase (eluent system B) and 100 μl were injected

The concentrations of the compounds in the samples were determined from their peak-height ratios relative to the internal standard and the corresponding least-squares line of the calibration standards

RESULTS AND DISCUSSION

Typical chromatograms obtained from plasma standards and patient samples are illustrated in Figs 2 and 3 Good separations were achieved under the con-

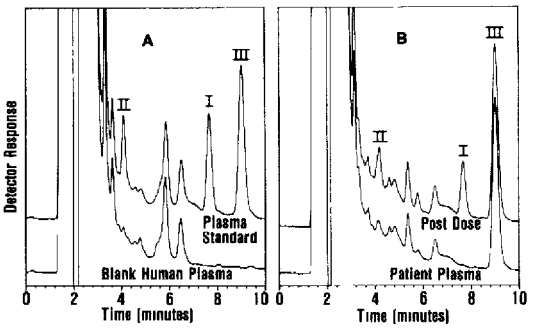

Fig 2 Chromatograms of plasma samples obtained by method A (A) Human plasma blank and a plasma standard containing 100 ng/ml pinacidil and 50 ng/ml pinacidil N-oxide (B) Patient plasma before dose and following a 12 5-mg dose of pinacidil monohydrate Peaks I = pinacidil, II = pinacidil N-oxide, III = internal standard

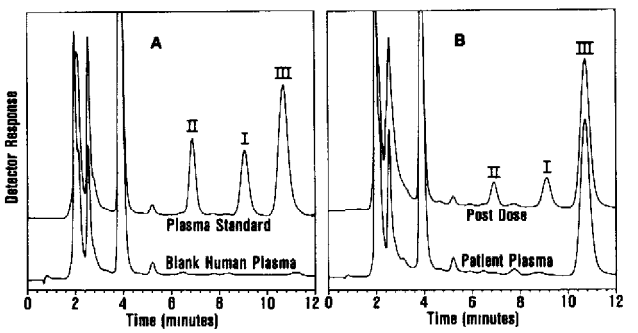

Fig 3 Chromatograms of plasma samples obtained by method B (A) Human plasma blank and a plasma standard containing 100 ng/ml each of pinacidil and pinacidil N-oxide (B) Patient plasma before dose and following a 12 5-mg dose of pinacidil monohydrate Peaks I = pinacidil, II = pinacidil N-oxide, III = internal standard

ditions in method A (Fig. 2) for both I and III from other sample components However, as shown in Fig 2, II was not completely resolved from other early eluting plasma constituents under these conditions This, combined with the low absorbance of II at 254 nm makes this procedure less than ideal for its determination except where concentrations are high The solid-phase extraction procedure utilized in method B provided a cleaner sample for analysis The mobile phase was adjusted to pH 4, where advantage can be made of the red shift in the UV spectrum of pinacidil with lower pH (Fig 4) The UV spectrum of II is not affected by pH (Fig. 5), therefore the two spectra overlap sufficiently to allow sensitive detection of both at 284 nm. Similarly, at pH 4, a decrease in the capacity factor, k', for I and III is observed but not for II As might be expected, the lower pH did cause a significant decrease in peak symmetry This tailing was effectively suppressed, however, by the addition of morpholine to the mobile phase.

Other drugs which may be concomitantly administered with pinacidil were tested for potential interference in the assay These included other antihypertensives, β-blockers, diuretics and other commonly administered drugs (Table I). The limited number of drugs tested for interference in method A is

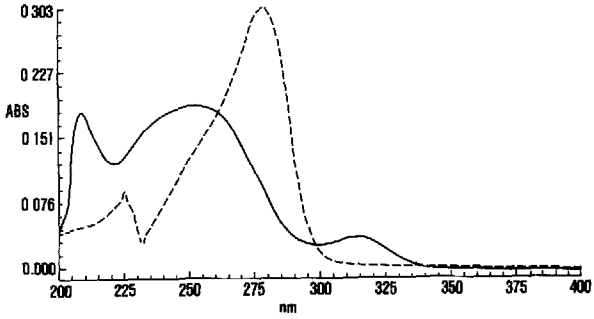

Fig 4 UV spectrum of 3 μg/ml pinacidil in 0 01 *M* ammonium phosphate, pH 7 0 (—), and 0 1 *M* sodium acetate, pH 4 0 (- - -)

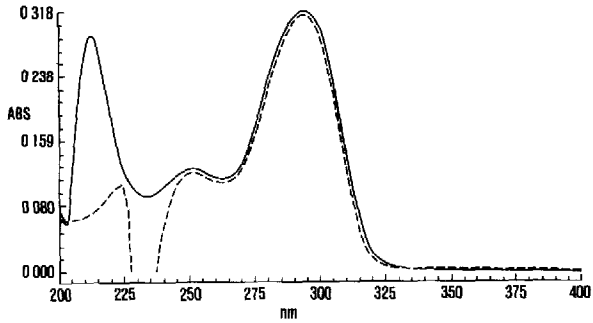

Fig 5 UV spectrum of 3 μg/ml pinacidil N-oxide in 0 01 *M* ammonium phosphate, pH 7 0 (—) and 0 1 *M* sodium acetate, pH 4 0 (- - -)

TABLE I

DRUGS TESTED FOR INTERFERENCE IN THE ASSAY

These drugs were tested for interference with the assay as stated Those marked with an asterisk present potential interference with the determination of pinacidil or pinacidil N-oxide

Method A	Method B
Hydralazine	Allopurinol
Hydrochlorothiazide	Atenolol
Prazosin*	Captopril
Propranolol	Chlordiazepoxide
Triamterene*	Clidinium bromide
	Clonidine
	Colchicine*
	Dipyridamole
	Furosimide
	Hydralazine
	Hydrochlorothiazide
	Metoprolol
	Minoxidil*
	Pilocarpine
	Prasozin*
	Propranolol
	Sulindac
	Triamterene

a result of our preferential use of method B Under the conditions employed in method A, triamterene interferes with the detection of pinacidil N-oxide and prasozin interferes with pinacidil Prasozin and pinacidil can be resolved from eacn other, however, by employing a mobile phase consisting of 0.01 *M* ammonium phosphate- methanol- acetonitrile (45 50 5).

Under the conditions employed in method B, potential interference with the determination of pinacidil N-oxide may result from the presence of prazosin and minoxidil Prasozin elutes before the N-oxide but may interfere if high concentrations are present Detection of minoxidil may be discriminated against by detection at 300 nm instead of 284 nm Colchicine coelutes with pinacidil but possesses a minimum in its absorbance spectra at 284 nm thus reducing the effect of its presence on apparent pinacidil plasma concentrations.

Sample recovery

The recovery of I, II and III from plasma was determined for both methods by a comparison of peak heights from spiked plasma prepared by each procedure to solutions of the analytes in mobile phase at equivalent concentrations For method A, the recovery of I and III was essentially 100% For method B, the recoveries of all analytes were greater than 90% for the concentrations tested (20—200 ng/ml).

Precision and accuracy

The precision and accuracy for each method was investigated by spiking blank plasma with I or I and II at four different concentrations Five replicates from each pool were assayed on two different days The concentrations ranged from 10 to 400 ng/ml I and from 5 to 200 ng/ml II The results are shown in

TABLE II

SUMMARY OF ACCURACY AND PRECISION DATA OF ASSAY FOR PINACIDIL (METHOD A)

Concentration (ng/ml)	*n*	Mean concentration found (ng/ml)	Relative standard deviation (%)	Relative error (%)
10	10	9 7	15 0	−3 0
30	10	30 2	6 8	+0 7
150	10	163 3	5 3	+8 9
400	10	430 6	1 8	+7 7

TABLE III

SUMMARY OF ACCURACY AND PRECISION DATA OF ASSAY FOR PINACIDIL (METHOD B)

Concentration (ng/ml)	*n*	Mean concentration found (ng/ml)	Relative standard deviation (%)	Relative error (%)
10	10	10 2	6 4	+1 0
30	10	39 7	1 9	−0 8
150	10	149 4	1 7	−0 4
400	10	401 1	1 0	+0 3

TABLE IV

SUMMARY OF ACCURACY AND PRECISION DATA OF ASSAY FOR PINACIDIL N-OXIDE (METHOD B)

Concentration (ng/ml)	n	Mean concentration found (ng/ml)	Relative standard deviation (%)	Relative error (%)
5	10	5 4	7 3	+8 0
20	10	19 9	4 5	−0 5
75	10	74 7	6 5	−0 4
200	10	204 7	6 7	+2 4

Tables II, III and IV. Relative standard deviations (R.S.D) for method A ranged from 15 to 1.8% for the determination of I The precision obtained over the same concentration range using method B was somewhat better, the R S D ranging from 6 4 to 1.0% Method B gave R S D. values for II from 7 3 to 4 5% over this concentration range The accuracy of these determinations is expressed in terms of percent relative error Good accuracy was observed for both methods, with values ranging from −3 to 9% by method A and −0 4 to 2% for method B for pinacidil Accurate determinations of pinacidil N-oxide (method B) are evident in relative errors of −4 to 8% obtained over the concentration range tested.

Linearity and sensitivity

The relationship between the concentrations of I and II and their peak-height ratios relative to the internal standard III was linear over a wide concentration range 5 ng/ml to at least 400 ng/ml by method B and 10 ng/ml to at least 400 ng/ml (I only) by method A The detection limit for pinacidil by method A was 10 ng/ml A slight improvement (5 ng/ml each) was observed in method B owing to improvements in selectivity and chromatography.

Stability

The stability of pinacidil and pinacidil N-oxide in plasma was determined by spiking a known amount of each compound into both human plasma and serum. Aliquots of these pools were stored in disposable glass centrifuge tubes, heparinized blood collection tubes, serum separation tubes and plastic sample shipping vials. The pools were analyzed for initial pinacidil and pinacidil N-oxide concentration and then at 24 h, one, two and four weeks and three months after storage at room temperature, 4°C, and −20°C Blood collection tubes were stored at room temperature only, with the tubes both upright and inverted to check for sample contamination from the stopper. No differences in stability were observed between plasma or serum pools, nor were any contaminants apparent from the stoppers of the blood collection tubes. Pinacidil was observed to be stable at room temperature in plasma for one month and for at least three months at −20°C. Losses of pinacidil N-oxide were apparent after one week at room temperature (20% loss), at 4°C (10% loss) and after four weeks following storage at −20°C (20% loss). No losses in pinacidil N-oxide concentrations were detected, however, after storage at −70°C for three months

Application of the method in pharmacokinetic studies

Eleven normal male volunteers were given 12 5-mg single oral doses of I in an open-label pharmacokinetic study. The dosage form employed in this study consisted of equal amounts of drug coated onto two different types of pellets One type promotes the immediate release of drug while the other delays its release for 4—6 h Blood samples were collected before the dose and at pre-determined time intervals after the dose. The concentration of I in the plasma was determined by method A The plasma profile obtained is illustrated in Fig. 6

In another open-label pharmacokinetic study, sixteen normal volunteers were given a single oral 12.5-mg dose of I Blood samples were collected in a similar fashion. Plasma concentrations of both I and II were determined by method B (Fig 7) The data obtained by these two methods were consistent between the two studies.

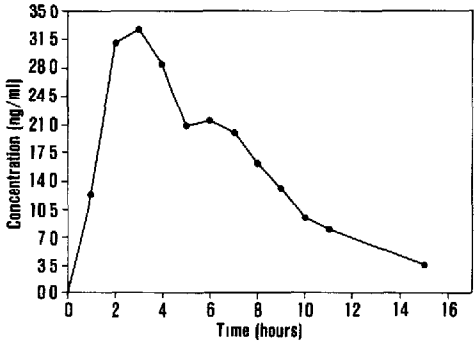

Fig 6 Profile obtained for pinacidil plasma concentrations versus time following a 12 5-mg dose of pinacidil monohydrate Samples were analyzed by method A, values reflect a mean of eleven patients

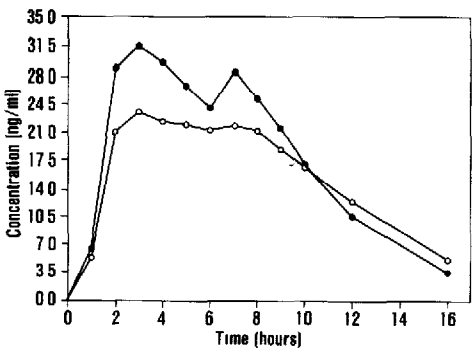

Fig 7 Profile obtained for pinacidil (•) and pinacidil N-oxide (○) plasma concentrations versus time following a 12 5-mg dose of pinacidil monohydrate Samples were analyzed by method B, values reflect a mean of sixteen patients

CONCLUSIONS

Pinacidil may be accurately and selectively determined in plasma by either of the two procedures described When metabolite concentrations are of interest, method B provides the necessary selectivity for the determination of both I and

II When parent drug concentrations are all that is required, method A may provide for a more cost-effective analysis as common solvents are employed instead of the more expensive solid-phase extraction cartridges. These savings may become apparent when large numbers of samples must be processed.

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