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SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ENDRALAZINE AND TWO OF ITS METABOLITES IN HUMAN PLASMA

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SUMMARY

Endralazine (I) is a new antihypertensive which is chemically and pharmacologically related to hydralazine and dihydralazine. A sensitive high-performance liquid chromatographic—fluorescence assay for the drug and two of its metabolites [methyltriazoloendralazine (VII) and hydroxymethyltriazoloendralazine (VIII)] in human plasma was developed. After conversion of I and its internal standard to triazolopyridopyridazine derivatives the latter and metabolites were separated by high-performance liquid chromatography and detected using their fluorescence. The limits of detection of the assay were 1 nmol/l for I and VII and 0.1 nmol/l for VIII. Intra-assay coefficients of variation were 2.5—5.1% for I (range 1000—10 nmol/l), 4.2—4.5% for VII (range 100—5 nmol/l) and 3.4—5.7% for VIII (range 100—1 nmol/l). Following oral administration of 5 and 10 mg of I to two normal volunteers (slow acetylators) peak plasma levels of I occurred between 0.75 and 1 h after the dose, and declined in a biexponential fashion. The terminal half-life ranged from 2.8—3.7 h. These results contrast with those obtained for hydralazine in plasma where in vitro and in vivo half-lives were < 30 min.

INTRODUCTION

Endralazine (6-benzoyl-3-hydrazino-5,6,7,8-tetrahydropyrido[4,3-*c*]pyridazine mesylate, I) is a new drug with antihypertensive properties [1—5] and is related to hydralazine and dihydralazine both chemically and pharmacologically. When given to man, a single oral dose of 10 mg of endralazine appears to elicit a response similar in magnitude to that obtained for approximately 50—100 mg of hydralazine [5].

Endralazine and hydralazine appear to undergo similar routes of metabolism, that is, acetylation, hydrazone formation and hydroxylation [5]. However,

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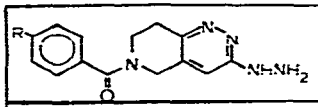
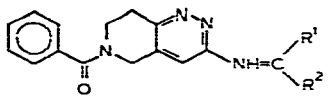
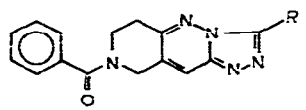
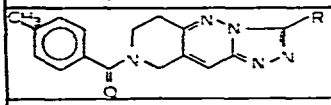
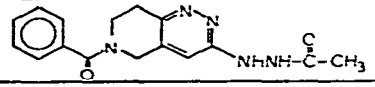
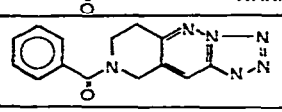
	I. R=H II. R=CH_3	Endralazine Endralazine internal standard
	III. R^1=CH_3, R^2=COOH IV. R^1=CH_2CH_2COOH, R^2=COOH V. R^1=CH_3; R^2=CH_3	Endralazine pyruvic acid hydrazone Endralazine α-ketoglutaric acid hydrazone Endralazine acetonide
	VI. R=H VII. R=CH_3 VIII. R=CH_2OH IX. R=CH_2CH_3	Endralazine derivative with formic acid Endralazine acetylation metabolite Endralazine acetylation metabolite Internal standard for VII
	X. R=H XI. R=CH_2OH	Endralazine internal standard derivative Internal standard for VIII
	XII.	Endralazine acetylation metabolite
	XIII.	Endralazine derivative with nitrous acid

these studies suggest that I may not exhibit the same acetylator phenotype determined differences in its metabolism which have been described for hydralazine [6]. Similarly, preliminary work [7] has suggested that I may not undergo the very rapid reaction with endogenous α-keto acids which has been described for hydralazine [6, 8].

It appears that to date there are no pharmacokinetic studies of I and its metabolites in humans reported. To this end, sensitive high-performance liquid chromatographic (HPLC) assays for I and the acetylation metabolites, VII and VIII, in human plasma were developed. The reaction rate of I with endogenous pyruvic acid in human plasma at 37°C in vitro and its pharmacokinetics following oral administration to two normal volunteers were studied using the method developed.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Chloroform was Nanograde from Mallinckrodt (St. Louis, MO, U.S.A.). Specially purified acetonitrile (210 nm cut-off, Unichrom from Ajax Chemicals, Melbourne, Australia) was used for HPLC. Endralazine mesylate (I), methylendralazine (II) the hydrazone metabolites (III and IV), and the acetylation metabolites (VII, VIII and XII) were provided by Sandoz (Basle, Switzerland). Although metabolite V has been identified [5], the pure substance was not available for these studies.

Standards

Stock solutions of I and II were prepared in methanol (200 μmol/l each) and stored in glass at 4°C. These solutions were prepared fresh weekly. Stock solu-

tions of the acetylation metabolites, VII and VIII (20 μmol/l of each) and their internal standards, IX (300 μmol/l) and XI (100 μmol/l) were also prepared in methanol and were stable for at least one month at 4°C. A solution containing I (10 μmol/l), VII and VIII (1 μmol/l of each) in water, was used to prepare the appropriate plasma standards at the time of each assay run or study. The internal standard mixture was also prepared fresh, prior to each assay run, and contained II (5 μmol/l), IX (7.5 μmol/l) and XI (2.5 μmol/l) in water.

Peak area and peak height ratios of the drug and metabolites to their internal standards, were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Syntheses

Synthesis of VI. 100 mg of I and 500 μl of 90% formic acid were heated at 90°C for 1 h in a loosely stoppered glass tube. The mixture was cooled, water added (1 ml) and the mixture neutralized with solid sodium bicarbonate. The precipitated solid was filtered, washed with distilled water and dried under vacuum at 20°C for 24 h. The solid had a melting point of 153—155°C and gave a single peak with retention time 4.2 min using the HPLC conditions described below. The electron impact mass spectrum determined at 20 eV, probe temperature 150°C using a Model AEI MS-30 mass spectrometer showed the molecular ion (*m/e* 279) and a fragmentation pattern consistent with structure VI.

Synthesis of internal standard XI. Methylendralazine (II) (50 mg) and 250 μl of 90% glycolic acid were heated at 90°C for 1 h in a stoppered glass tube. The mixture was cooled and neutralized with solid sodium bicarbonate. The precipitated solid was filtered, washed with water and dried under vacuum at 21°C. The solid had a melting point of 216—217°C and gave a single peak with retention time 26.3 min using the HPLC conditions described below. The field desorption mass spectrum was consistent with the structure of the glycolic acid ester of XI and showed a molecular ion with mass 381. This ester (850 μg) was readily and completely hydrolyzed to XI by treatment with 1 ml of 1 *N* aqueous sodium hydroxide at 21°C for 2 h. The solution was then neutralized with hydrochloric acid and diluted to 100 μmol/l with water and used as the stock solution (stable for at least one month at 4°C). It gave a single peak with retention time 5.8 min by HPLC.

Synthesis of internal standard IX. Fifty milligrams of I and 250 μl of 90% propionic acid were heated at 90°C for 1 h in a stoppered glass tube. The mixture was cooled, diluted with water (0.5 ml) basified (pH 12) with 1 *N* aqueous sodium hydroxide and extracted with chloroform (5 ml). The chloroform was evaporated under nitrogen at 45°C leaving an oil which slowly solidified. Using the HPLC conditions described below the solid gave a single peak with retention time 14.0 min. The electron impact mass spectrum determined at 20 eV with a probe temperature of 170°C showed the molecular ion (*m/e* 307) and a fragmentation pattern consistent with structure IX.

Reaction of I with formic acid

To optimize the conversion of I to the fluorescent derivative VI the conditions of the reaction with formic acid were closely studied. A methanolic solu-

tion of I was prepared (10 μmol/l) and 100 μl (containing 1 nmol of I) aliquoted into glass culture tubes (diSPo tubes from Scientific Products, McGaw Park, IL, U.S.A.) (75 × 12 mm) and the methanol removed under a stream of pure nitrogen at 45°C. A 50-μl aliquot of 90% aqueous formic acid was added to the tubes and they were sealed with firmly fitting plastic caps. Pairs of tubes were incubated at room temperature (21°C) and at 80 and 90°C in a heating block for 0.5 h. Other tubes were incubated at 90°C and pairs removed 10, 15, 30, 45 and 60 min after commencement of incubation. A 450-μl aliquot of mobile phase, 1.5 m*M* phosphoric acid—acetonitrile (82:18) was added to each tube and 100 μl then injected into the chromatograph. The peak area of VI was compared with that of a known amount of synthesized material injected into the chromatograph.

Blood collection and assay procedure

Venous blood samples (10 ml) were drawn into plastic syringes and transferred to ice cold polypropylene tubes containing 125 I.U. of lithium heparin. The blood was then aliquoted into conical polypropylene tubes (TC-10 centrifuge tube from Medical Plastics, Melbourne, Australia) and centrifuged at 8000 *g* in an Eppendorf Model 5412 centrifuge for 30 sec. The plasma was immediately transferred to an ice cold polypropylene tube. Plasma (1 ml) was transferred to a 15-ml glass stoppered tube; 100 μl of the internal standard mixture containing II (5 μmol/l), IX (7.5 μmol/l) and XI (2.5 μmol/l) were added followed by 5 ml of chloroform. Extraction was carried out immediately by vortexing for 30 sec and the phases were separated by centrifugation (5 min at 1100 *g*). The aqueous layer was removed by vacuum aspiration and discarded, and the organic layer was poured into autosampler tubes (75 mm × 12 mm, diSPo tubes from Scientific Products), and evaporated at 45°C under a stream of pure nitrogen. 90% formic acid (50 μl) was added and the mixture was vortexed gently. The tubes were then tightly capped and incubated at 90°C in a heating block for 30 min. The formic acid was evaporated at 90°C under a stream of pure nitrogen, 1 drop of ammonia added to hydrolyse the formic acid esters of the hydroxymethyltriazolo compounds and then this was also evaporated. The residue was reconstituted in mobile phase, 1.5 m*M* phosphoric acid—acetonitrile (82:18) and 100 μl injected into the chromatograph.

High-performance liquid chromatography

The chromatograph used (Spectra-Physics Model SP 8000) was equipped with a ternary solvent system, helium degas and automatic data reduction facilities. A 10-μm RP-8 reversed-phase column (Spectra-Physics, Santa Clara, CA, U.S.A.) was used at a column temperature of 55°C. The instrument was operated in the constant flow mode and the mobile phase consisted of 1.5 m*M* aqueous phosphoric acid—acetonitrile (82:18) with a flow-rate of 2 ml/min.

All solvent lines from the column to the detector were carefully thermally insulated. The column effluent was monitored using a fluorescence detector (Schoeffel, Model 970) at an excitation wavelength of 230 nm with an emission cut-off filter allowing 90% transmission at 389 nm. The fluorimeter sensitivity was 3.5, range 0.1 μA full scale and time constant 6.0 sec. Samples were injected automatically using a 100-μl injector loop and an autosampler (Spec-

tra-Physics Model 8010). All files for instrument operation and integration were stored on disc (Spectra-Physics Model SP 4010 disc module).

Reproducibility and recovery

Intra-assay reproducibility for the endralazine and metabolite assay was determined by assaying five replicate plasma samples containing added amounts of drug and metabolites at concentrations ranging from 0.1—1000 nmol/l (Table I). Day-to-day reproducibility was not determined directly because of the in vitro reaction of the parent drug with α-keto acids in plasma which could occur during freezing and thawing of samples. This precluded storing a bath of frozen samples and assaying one within each assay run. An estimate of day-to-day precision was obtained by examining the variability in the slope of the standard curves for ten consecutive runs on different days.

Recoveries of I, VII and VIII from plasma in the assay method were determined by injecting known amounts of the endralazine derivative (VI) and the metabolites (VII and VIII) into the chromatograph and comparing the peak areas with those obtained for plasma standard of known concentration.

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE ASSAY OF ENDRALAZINE AND METABOLITES

Five replicate determinations at each concentration.

Drug/metabolite	Coefficient of variation (%) at concentrations (nmol/l)						
	1000	100	50	10	5.0	1.0	0.1
I	2.5	1.2	2.7	5.1	—	17.3	—
VII	—	4.2	—	4.0	4.5	18.6	—
VIII	—	3.4	—	—	4.8	5.7	19.8

Specificity of the HPLC assays

The endralazine hydrazones (III and IV) and the uncyclized acetylation metabolite (XII) were added to plasma to give a final concentration of 10 μmol/l and carried through the assay procedure described above. If conversion to either the endralazine derivative (VI) or to the metabolites VII and VIII occurred, the percentage converted was determined in each case. In addition, a number of fluorescent drugs and their metabolites (Table II) were added to plasma and subjected to the conditions of the assay procedure. If a peak was obtained the retention time was recorded.

Stability of extracted samples

To determine whether any change occurred in samples after chloroform extraction, standards containing I, VII and VIII in a concentration of 100, 10 and 10 nmol/l respectively were extracted with chloroform as described above. One pair of samples (duplicates) was immediately processed as described above and injected into the chromatograph. Additional pairs were allowed to stand for 2 and 4 h after vortexing without centrifugation and then processed as

TABLE II

RETENTION TIMES OF A NUMBER OF FLUORESCENT DRUGS AND THEIR METABOLITES

Drug	Retention time (min)
Endralazine metabolite VIII	3.2
Endralazine derivative VI	4.2
Internal standard XI	5.8
Endralazine metabolite VII	7.0
Internal standard derivative X	8.7
Internal standard IX	14.0
3-Hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine*	2.3
s-Triazolo[3,4-*a*]phthalazine*	3.3
3-Methyl-*s*-triazolo[3,4-*a*]phthalazine*	5.3
4-Hydroxypropranolol**	25.5
N-Desisopropylpropranolol**	46.7
Propranolol	112
Atenolol	NP***
Metoprolol	NP
Timolol	NP
Frusemide	6.0
Methyldopa	NP
Prazosin	NP

*Hydralazine metabolites.
**Propranolol metabolites.
***No peak observed with a retention time less than 2 h.

before. These samples were then injected into the chromatograph and peak area ratios to the internal standards obtained for each sample.

Reaction of I in plasma in vitro at 37°C

Fresh venous blood from a non-medicated normal volunteer was heparinized and centrifuged immediately. The plasma pyruvic acid level was estimated using an enzyme assay kit (Boehringer diagnostic kit). To half of the plasma was added endralazine to provide a final concentration of 1.0 μmol/l and the mixture maintained at 37°C in a water bath. Samples (1 ml) were taken at times 0, 5, 10, 15, 30, 45 min and 1.0, 1.5, 2.0, 2.5 and 3 h after the addition of I and were assayed for I, VII and VIII by the method described. To compare the rates of reaction of I and hydralazine at 37°C, a duplicate experiment was carried out simultaneously with 1.0 μmol/l of hydralazine using the same plasma. Levels of hydralazine were determined by HPLC using the method described previously [9].

Pharmacokinetic study

Two slow acetylators of sulphadimidine [10] were each given 5-mg and 10-mg oral doses of endralazine with 500 ml of water, on separate occasions more than one week apart. Blood samples were drawn 0, 10, 20, 30, 40, 50 min and 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 h after the dose and the plasma assayed for I, VII and VIII using the method described.

RESULTS

HPLC assay for endralazine and metabolites

Chromatograms obtained for the assay of I, VII and VIII are shown in Fig. 1. Intra-assay coefficients of variation are shown in Table I. Recovery of I was 84% over the concentration range 1.0—1000 nmol/l, 92% for VII over the range 1.0—100 nmol/l and 85% for VIII over the range 0.1—100 nmol/l. The variation in the slope of the standard curves was 3.5%, 5.6% and 9.9% for I, VII and VIII, respectively. Limits of detection (determined at peak height twice noise) were 1 nmol/l (0.3 ng/ml) for I and VII and 0.1 nmol/l (0.03 ng/ml) for VIII following injection of half the extracted sample on column. No change in peak areas or heights was observed in extracted samples which were allowed to stand for 2 and 4 h before centrifugation and removal of the chloroform layer. Similarly no change occurred over 24 h in derivatized samples reconstituted in mobile phase and allowed to stand at 21°C.

Reaction of I and the internal standard II with aqueous formic acid at 90°C for 30 min resulted in complete conversion to the respective fluorescent derivatives VI and X. Shorter derivatization times or lower temperatures resulted in reduced recovery of VI. Other approaches to the derivatization of I using trifluoroacetic anhydride, propionic anhydride, dansyl chloride, *o*-phthalaldehyde and formaldehyde were unsuccessful.

Specificity

At a level of 10.0 μmol/l the endralazine hydrazones III and IV interfered in the estimation of I to the extent of 0.7% and 0.9%, respectively. The un-

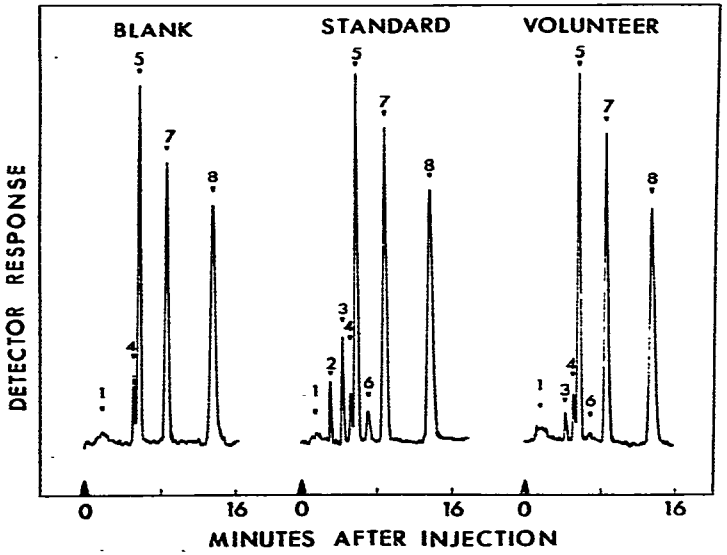


Fig. 1. Chromatograms obtained for the assay of endralazine and metabolites in blank plasma, in a plasma standard containing 100 nmol/l of endralazine and 10 nmol/l of VII and VIII and in plasma from a volunteer following an oral dose of endralazine (5 mg) and containing 26 nmol/l of I and 1.4 nmol/l of VIII. Peaks: 1 = plasma peak; 2 = VIII; 3 = VI; 4 = plasma peak; 5 = XI; 6 = VII; 7 = X; 8 = IX.

cyclized acetyl metabolite XII interfered in the assay of I to the extent of 4.5% and in the assay of VII to the extent of 5.1%. Since plasma levels of XII are apparently very low [5] this represents insignificant interference in the assay. However, longer derivatization times with formic acid at 90°C resulted in increased conversion of XII to both VI and VII. The retention times of other drugs and metabolites carried through the assay procedure are summarized in Table II.

Reaction of I in plasma in vitro at 37°C

I did not react as rapidly as hydralazine with endogenous pyruvic acid in plasma in vitro at 37°C. The first order half-life for hydralazine was 11.0 min compared with 3.8 h for I (Fig. 2).

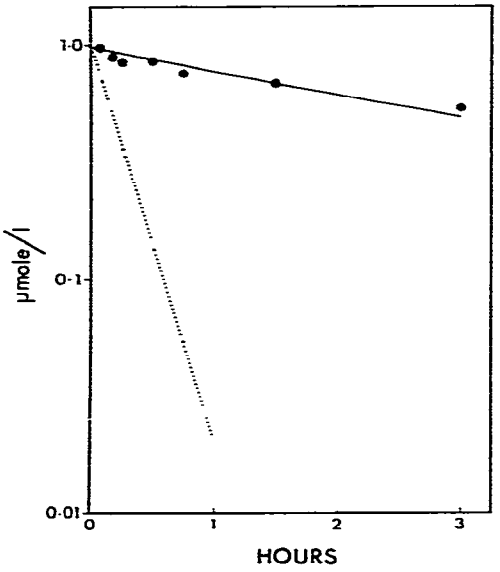


Fig. 2. Plasma level—time course of endralazine and hydralazine (1 µmol/l of each) following their addition to fresh plasma at 37°C containing endogenous pyruvic acid (88 µmol/l). ●——● = I; o • • • o = hydralazine.

DISCUSSION

Endralazine (I) and two of its metabolites, VII and VIII were extracted in good recovery from plasma at physiological pH and separated by HPLC. Separate internal standards were used for each component, and I and its internal standard (II) were selectively derivatized with formic acid to yield the triazolopyridopyridazines, VI and X. The latter and VII and VIII were detected by fluorescence allowing levels less than 1 nmol/l to be measured in plasma. No significant interferences by known metabolites of I [5] and other fluorescent drugs and their metabolites were observed in the assays. Extraction of plasma samples without subsequent derivatization demonstrated that VI was not a naturally occurring metabolite of I in the plasma of healthy volunteers. Repro-

ducibility of quantification at low plasma levels was sufficient for single-dose pharmacokinetic studies.

Attempts to extend the assay methodology developed for hydralazine [9] to the assay of I were unsuccessful. In this case the tetrazolo derivative of I (XIII) formed by treatment with weak nitrous acid was insufficiently fluorescent to allow small plasma levels to be measured. Endralazine itself, the hydrazone metabolites (III and IV) and the uncyclized acetyl metabolite (XII) had no inherent fluorescence under the chromatographic conditions presently described.

Although endralazine reacted with pyruvic acid in plasma in vitro at 37°C, the rate of this reaction was considerably slower than for hydralazine under identical conditions (Fig. 2). At 4°C the reaction of I in freshly drawn plasma was sufficiently slow (half-life = 23 h) that samples could be stored for up to 1 h without significant losses of I (< 1%). This allowed a number of samples to be drawn rapidly in pharmacokinetic studies and to be extracted as a group after storage at 4°C for up to 1 h.

Following oral administration of 5 and 10 mg of endralazine to two slow acetylators, plasma levels of I peaked between 0.75 and 1.0 h after the dose and then declined biexponentially (Fig. 3). Terminal half-lives ranged from 2.82 to 3.76 h and did not appear to be affected by dose. The ratios of the area under the plasma level—time curve from time zero to infinity (AUC_0^∞) of I for the 10- and 5-mg doses were 1.8 and 1.9 in the two subjects. Plasma levels of the acetylation metabolite VII were detected in both subjects but did not exceed 2 nmol/l. Metabolite VIII was not detected in plasma and prior hydrolysis of its conjugates may be necessary before detection is possible.

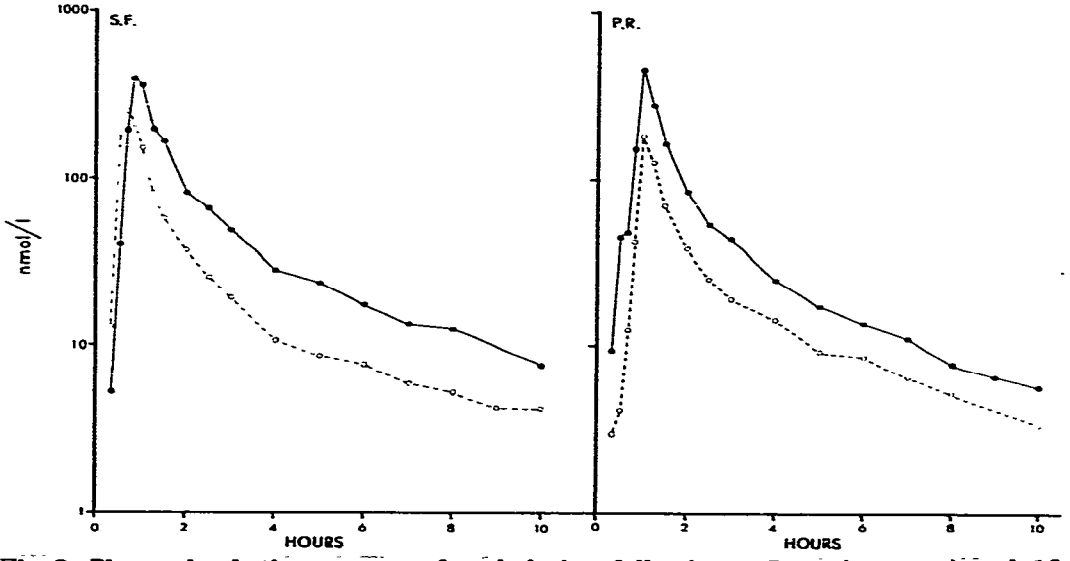


Fig. 3. Plasma level—time course of endralazine following a 5-mg (o • • • o) and 10-mg (•——•) oral dose of endralazine to two slow acetylators.

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