Journal of Chromatography, 181 (1980) 427—440
Biomedical Applications

CHROMBIO. 496

SELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS FOR HYDRALAZINE AND ITS METABOLITES IN PLASMA OF MAN

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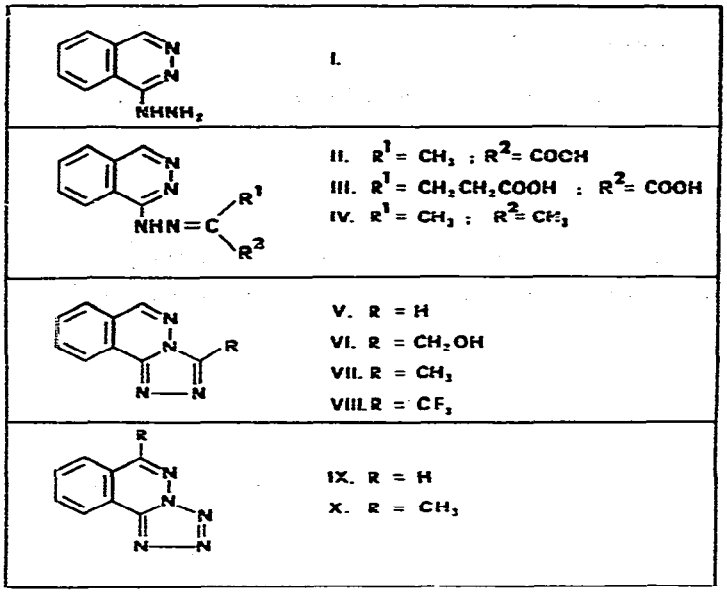
(First received September 3rd, 1979; revised manuscript received November 12th, 1979)

SUMMARY

Selective high-performance liquid chromatographic assays for hydralazine (I), hydralazine pyruvic acid hydrazone (II) and the acetylation metabolites, namely *s*-triazolo[3,4-*a*]-phthalazine (V) and 3-hydroxymethyl (VI) and 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (VII) in human plasma were developed. Utilizing the fluorescence of these compounds or their derivatives the limits of detection could be extended down to 5 nmole/l (1 ng/ml) for I, 1 nmole/l (0.2 ng/ml) for II and 0.5 nmole/l (0.1 ng/ml) for V—VII. The intra-assay coefficients of variation for the assays ranged from 2 to 7% over the concentration range 5.0 to 0.05 μmole/l and the inter-assay variability in the slope of the standard curves ranged from 4 to 8%. An improved method for measuring the sum of I plus all its hydrazones (apparent I) was also developed. On addition of I to fresh plasma at 37°, half the added I was converted to II within 15 min and there was no detectable level of I, 2 h after the addition. The plasma level—time course of I, and its metabolites in a healthy volunteer (slow acetylator) following separate oral and intravenous administrations of I indicated that I contributed only a small fraction (4.3 and 4.7% respectively) to the area under the plasma level—time curve of apparent hydralazine.

INTRODUCTION

With the "comeback" of hydralazine (I) [1] there has been renewed interest in the measurement of plasma levels of the drug and its metabolites in man. Consequently, several plasma assay methods have been reported [2—4] together with determined pharmacokinetic parameters [5—10]. However, it has been convincingly demonstrated that the available assay methods lack specificity for unmetabolized hydralazine [11—13]. Acid labile hydrazones are converted back to hydralazine by the acidic conditions of the derivatization procedures and it is an "apparent hydralazine" level which is obtained. Two modifications of the original gas—liquid chromatographic assay have been reported



[13, 14] and one of these [13] still lacked sufficient specificity [15] for accurate determinations of I. Many of the pharmacokinetic parameters determined using the non-specific hydralazine assays are therefore misleading.

We have shown that the major hydrazone present in plasma is hydralazine pyruvic acid hydrazone (II) [12], formed by chemical reaction between hydralazine and endogenous pyruvic acid and accounts for more than 90% of apparent hydralazine levels at steady-state. Other hydrazones (III and IV) have also been identified in plasma but not quantitated [16].

In the present report specific high-performance liquid chromatographic (HPLC) assays for the parent drug (I), its metabolites (II, V—VII) and apparent hydralazine in plasma are described. I and II were measured after conversion to the fluorescent tetrazolo- and triazolo[3,4-*a*]phthalazine derivatives, respectively, and V—VII detected using their native fluorescence.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Specially purified acetonitrile (Unichrom from Ajax Chemicals, Melbourne, Australia) was used for HPLC. The synthesis of the hydralazine metabolites and 3-trifluoromethyl-*s*-triazolo[3,4-*α*]phthalazine (VIII) has been described previously [12]. 4-Methylhydralazine was generously donated by Ciba-Geigy, Basle, Switzerland.

Standards

Stock solutions of hydralazine hydrochloride (I, 50 μmole/l) and the internal standard, 4-methylhydralazine (16.7 μmole/l) were prepared in 0.01 *N* aqueous hydrochloric acid and stored in glass at 4°. These solutions were prepared fresh

weekly. Solutions of the hydrazones (II, III and IV) were prepared in 0.01 *N* aqueous sodium hydroxide and prepared fresh prior to each assay run or study. A stock solution containing the metabolites V, VI and VII (5.0 μmole/l each) was prepared in 0.01 *N* hydrochloric acid and was stable for several months at 4°. The internal standard for the assay of the metabolites V—VII, 3-trifluoromethyl-*s*-triazolo[3,4-*a*]phthalazine (VIII) was prepared in 0.01 *N* hydrochloric acid (5 μmole/l) and was also stable for several months at 4°.

Plasma standards of I, II and V—VII were prepared using the above solutions at the time of each assay run. In the case of I and II, derivatization was performed immediately after the addition of the standard and internal standard solutions to plasma to avoid losses due to reaction with endogenous keto-acids present in plasma.

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

Blood collection and plasma treatment

Special procedures were necessary and were adopted when plasma concentrations of circulating levels of hydralazine were measured. Venous blood samples (8—12 ml) were drawn quickly into plastic syringes and immediately transferred to ice-cold polypropylene tubes containing 125 I.U. of lithium heparin. The blood was then aliquoted into polypropylene conical centrifuge 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 a polystyrene tube kept in ice and two 1-ml aliquots put into separate glass tubes each containing 75 μl of 50% aqueous sodium nitrite. These samples were then immediately treated in the way described for the assay of I and II respectively. The entire procedure from the time of drawing the blood to the time of derivatization should be performed in less than 5 min. The remainder of the plasma was stored at −20° and assayed for apparent hydralazine and metabolites V—VII within 24 h.

High-performance liquid chromatography

The chromatograph used (Spectra-Physics Model SP 8000) was equipped with a ternary solvent system, helium degass and automatic data reduction facilities. A 10-μm alkyl phenyl reversed-phase column (μBondapak/phenyl from Waters Assoc., Milford, Mass., U.S.A.) was used at a column temperature of 50°. The mobile phase was 1.5 m*M* aqueous phosphoric acid—acetonitrile (either 85 : 15 or 80 : 20) at a flow-rate of 2 ml/min and 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 250 nm with an emission cut-off filter allowing 90% transmission at 360 nm. The fluorimeter sensitivity setting was 3.5, range 0.1 μA full-scale and time constant 4.0 sec. Samples were injected automatically using an autosampler (Spectra-Physics Model 8010). All files for the operation of the chromatograph and for the processing of raw data for each assay were stored on disc (Spectra-Physics Model 8040) and recalled as required.

HPLC assay of I

To one of the tubes containing plasma and sodium nitrite described above, was added the internal standard solution (4-methylhydralazine, 150 μl of 16.7 μmole/l) and 2 ml of 0.02 *N* aqueous hydrochloric acid to give a final pH of 5.5. The mixture was then briefly vortexed and allowed to stand for exactly 10 min at 20 ± 1°. The entire procedure from the time of drawing the blood to the time of addition of the acid must be performed in less than 5 min. To the acidic mixture was then added 1 ml of an aqueous 1 *N* sodium hydroxide—0.6 *M* sodium tetraborate solution (pH 10) followed by chloroform (Nanograde from Mallinckrodt, St. Louis, Mo., U.S.A.). Extraction was carried out by shaking at 110 rpm for 5 min. The phases were separated by centrifugation (10 min at 1100 *g*) and the aqueous layer removed by vacuum aspiration. The organic layer was poured into culture tubes (diSPo tubes from Scientific Products, McGaw Park, Ill., U.S.A.) (75 × 12 mm) and evaporated under a stream of pure nitrogen at 45°. The residue was reconstituted in 0.5 ml of mobile phase consisting of 1.5 m*M* aqueous phosphoric acid—acetonitrile (85 : 15) and 50 μl injected into the chromatograph.

HPLC assay of II

The method employed for the assay of metabolite II was identical to that described for hydralazine itself except that plasma standards and samples containing II were treated with 0.1 *N* hydrochloric acid rather than 0.02 *N* providing a final pH of 3.9. Again the entire procedure from the time of drawing the blood to the time of acid treatment should be performed in less than 5 min. The level determined by this procedure included the acetylated material VII which was quantitated separately. The level of II was then determined by subtraction.

HPLC assay of V, VI and VII

To 1 ml of heparinized plasma in a glass tube was added the internal standard solution (VIII, 150 μl of 5.0 μmole/l) followed by 1 ml of an aqueous 1 *N* sodium hydroxide—0.6 *M* sodium tetraborate solution (pH 10). Five ml of chloroform were added and the mixture shaken at 100 rpm for 5 min. The phases were separated by centrifugation (10 min at 1100 *g*) and the aqueous layer removed by vacuum aspiration. The organic layer was poured into culture tubes and evaporated as before. The residue was dissolved in 0.5 ml of mobile phase consisting of 1.5 m*M* aqueous phosphoric acid—acetonitrile (80 : 20) and 50 μl injected into the chromatograph.

HPLC assay of apparent hydralazine

The method was a modification of the method of Jack et al. [4]. To 1 ml of heparinized plasma was added the internal standard solution (4-methylhydralazine, 150 μl of 16.7 μmole/l) followed by 2 ml of 2 *N* hydrochloric acid and 200 μl of 50% aqueous sodium nitrite solution. The mixture was vortexed and allowed to stand at 20 ± 1° for 15 min. An amount of 2.5 ml of 4 *N* aqueous sodium hydroxide was then added followed by 5 ml of chloroform. Extraction was carried out by shaking at 100 rpm for 5 min and the phases were separated by centrifugation (10 min at 1100 *g*). The aqueous layer was removed by

vacuum aspiration and the organic layer poured into culture tubes and evaporated as before. The residue was reconstituted in 1 ml of mobile phase [1.5 m*M* aqueous phosphoric acid—acetonitrile (85 : 15)] and 50 μl injected into the chromatograph.

Reproducibility and recovery

The intra-assay reproducibilities for the hydralazine and metabolites assays were determined by assaying five replicate plasma samples containing added amounts of drug and metabolites at concentrations ranging from 0.05—5.0 μmole/l.

Inter-assay reproducibility was not determined directly because of the rapid reaction of the parent drug with α-keto acids in plasma which precluded storing a batch of frozen samples and assaying one within each assay run. However the variation in the slopes of the standard curves for each assay was determined for five consecutive runs on different days. Recoveries of the methods were determined by injecting known amounts of IX and V—VII into the chromatograph and comparing the peak areas with those obtained for plasma standards of known concentration.

Specificity of the HPLC assays

The hydralazine hydrazones (II—IV) were added to plasma to give a final concentration of 5.0 μmole/l and assayed for hydralazine by the method described above. If conversion of the hydrazones to I occurred, the percentage converted was determined in each case. The hydralazine metabolites (V—VII) and a number of relevant and representative fluorescent drugs and their metabolites propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol, quinidine, dihydroquinidine, 3-hydroxyquinidine, imipramine and desipramine were added to plasma and subjected to the conditions of the hydralazine and hydralazine pyruvic acid hydrazone assays. If a peak was obtained the retention time was recorded. Similarly, the hydrazones II—IV and propranolol, quinidine, imipramine and their metabolites were added to plasma and checked for interference in the assay for V—VII.

Stability of derivatized plasma samples to storage

I and II were added to plasma to provide a concentration of 5.0 μmole/l each and 1-ml aliquots placed in polystyrene tubes containing 75 μl of 50% aqueous sodium nitrite. The internal standard, followed by 2 ml of 0.1 *N* hydrochloric acid were then added and the tubes allowed to stand at 20 ± 1° for 10 min. An amount of 1.0 ml of 1 *N* aqueous sodium hydroxide—0.6 *M* sodium tetraborate (pH 10) was then added to all tubes and of these, five were immediately extracted with chloroform in glass tubes and assayed for I and II. Of the remaining tubes, five were left at 20 ± 1° and extracted 24 h later. Another five were stored at 4° for 4 h before extraction and assay. The remainder were frozen and stored at −20°. A number of these tubes were thawed 24 h, 1 week and 2 weeks after the initial treatment and assayed as before.

Reaction of I in plasma in vitro at 37°

Fresh venous blood from a non-medicated normal volunteer was heparinized

and centrifuged immediately. To the plasma was added hydralazine to provide a final concentration of 1.1 μmole/l and the mixture maintained at 37° in a water bath. Samples (1 ml) were taken at times 5, 10, 20, 30, 40, 60, 90 and 120 min after the addition and assayed for I, II and apparent hydralazine by the methods described. Measurement of I was also performed using 0.1 *N* hydrochloric acid treatment in order to compare with the results obtained using 0.02 *N* acid.

Pharmacokinetic studies

A healthy volunteer (87 kg) was acetylator phenotyped [17] and given an oral (1 mg/kg) and an intravenous (0.375 mg/kg) dose of hydralazine on separate occasions three weeks apart. The approximate oral dose (correct to the nearest 5 mg) was made up of a suitable combination of whole or fractions of 50- and 25-mg Apresoline® tablets and was administered with 150 ml of water. Blood samples were drawn at times 0, 10, 20 min and 0.5, 0.75, 1.0, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h after the dose. The exact intravenous dose (Apresoline® for Injection) was diluted in 20 ml of sterile water for injection and administered as an infusion over 5 min. Blood samples were drawn from an ante cubital vein at times 0, 5, 10, 15, 20, 25 min and 0.5, 0.75, 1.0, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 7 and 10 h after commencement of the infusion. All blood samples were treated in the manner described earlier and assayed for I, II and V—VII.

RESULTS

HPLC assays for hydralazine and metabolites

Chromatograms obtained for the assays of I, II and V—VII and apparent hydralazine are shown in Figs. 1—3. The intra-assay coefficients of variation for the assays are shown in Table I, variations in the slope of the standard curves in Table II and recoveries for each of the assays in Table III. The limits of detection (determined at peak height twice noise) were 5 nmole/l (1 ng/ml) and 1 nmole/l (0.2 ng/ml) for I and II respectively and 0.5 nmole/l (0.1 ng/ml) for metabolites V—VII.

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE ASSAYS OF HYDRALAZINE AND METABOLITES

Drug/metabolite	Coefficient of variation (%) at concentrations* (μmole/l)						
	5.0	2.5	1.25	0.5	0.25	0.125	0.05
I	2	3	3.5	3.5	–	–	4
II	3	3	3	3.5	–	–	7
V	–	–	–	3	2	3	3
VI	–	–	–	2	4	3	2
VII	–	–	–	3	2	3	2

*Five replicate determinations at each concentration.

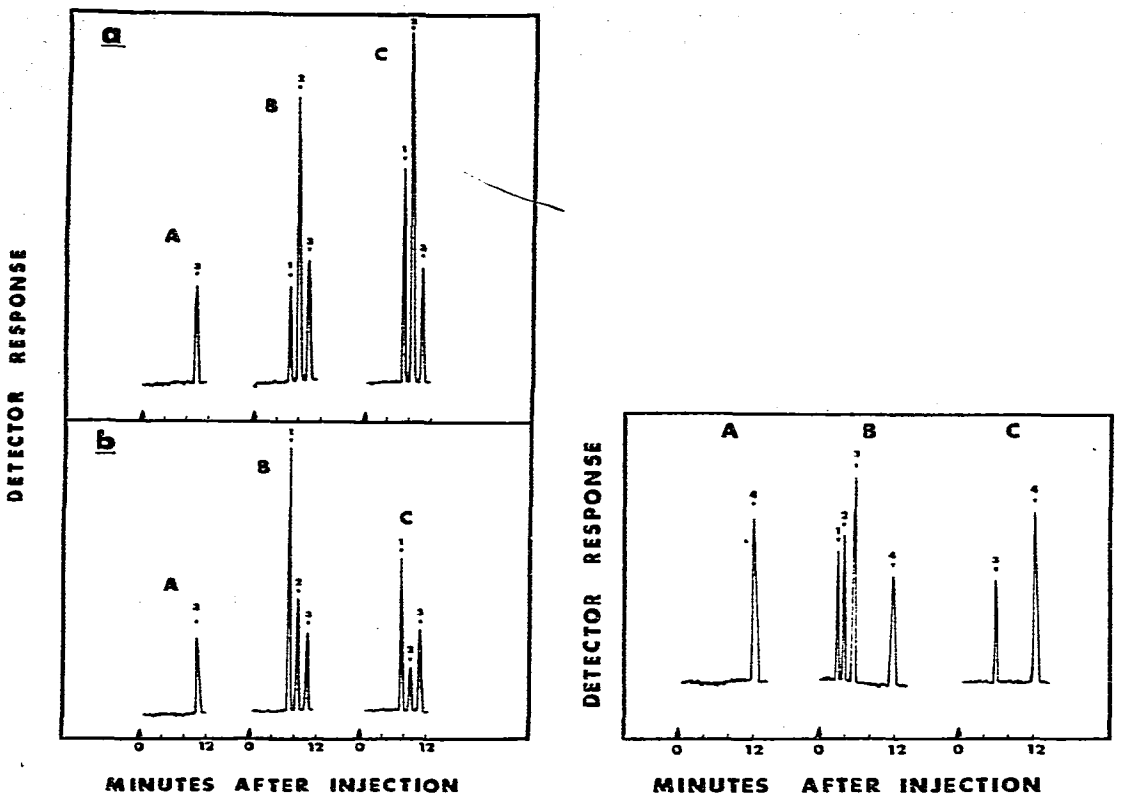


Fig. 1. Chromatograms obtained for the assay of I and II at (a) pH 3.9 and (b) pH 5.5. A = blank plasma; B = plasma standard containing I and II (1.25 and 5.0 μmole/l for a and b respectively); C = plasma from a volunteer following an intravenous dose of hydralazine (0.375 mg/kg) and containing I (2.9 μmole/l) and II (1.4 μmole/l). Peaks: 1 = IX, derivative of I; 2 = VII, derivative of II; 3 = X, derivative of the internal standard.

Fig. 2. Chromatograms obtained for the assay of the hydralazine metabolites V—VII. A = blank plasma; B = plasma standard containing 0.25 μmole/l each of V, VI and VII; C = plasma from a volunteer following an oral dose of hydralazine (1 mg/kg) and containing VII. Peaks: 1 = VI; 2 = V; 3 = VII; 4 = internal standard VIII.

TABLE II

VARIATION IN THE SLOPE OF THE STANDARD CURVES FOR THE HYDRALAZINE AND METABOLITE ASSAYS

Drug/metabolite	Coefficient of variation* (%)
I	8
II	8
V	5
VI	6
VII	6
Apparent hydralazine	4

*Determined from five consecutive standard curves on different days for each assay.

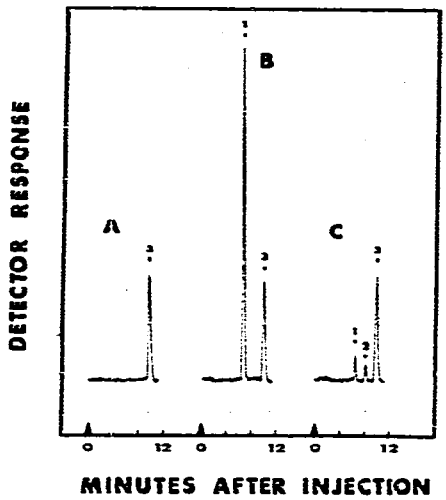


Fig. 3. Chromatograms obtained for the assay of apparent hydralazine, i.e. hydralazine plus hydrazones. A = blank plasma; B = plasma standard containing 5.0 μmole/l of I; C = plasma from a volunteer following an oral dose of hydralazine (1 mg/kg) and containing 0.34 μmole/l of apparent hydralazine. Peaks: 1 = IX; 2 = VII; 3 = X.

TABLE III

RECOVERIES FOR THE HYDRALAZINE AND METABOLITE ASSAYS

Drug/metabolite	Concentration range (μmole/l)	Recovery (%)
I	1.25 — 5.0	75
II	1.25 — 5.0	88
V	0.125— 0.5	98
VI	0.125— 0.5	60
VII	0.125— 0.5	98
Apparent hydralazine*	1.25 —10.0	86

*Recovery of I or II added to plasma.

Nitrous acid generated using 0.02 *N* hydrochloric acid (pH 5.5) converted more than 75% of the hydralazine in plasma to the derivative IX but only partly converted II to VII (17% recovery). Nitrous acid generated using 0.1 *N* hydrochloric acid (pH 3.9) resulted in almost 90% conversion of II to VII and after subtraction of the endogenous plasma level of VII allowed accurate quantitation of II. However, reduced accuracy can be expected if levels of VII are much greater than II. The conversion of II to VII was confirmed by gas chromatography—mass spectrometry where the spectrum obtained for the product was identical to that of an authentic sample of VII. Although no conversion of II to IX occurred, the stronger acid conditions (pH 3.9) converted 70% of III and 5% of IV added to plasma (5.0 μmole/l) to IX and therefore interfered in the determination of the parent drug. Separate conditions were therefore required for the estimation of I and II, that is, 0.02 *N* and 0.1 *N* hydrochloric acid providing pH 5.5 and 3.9 respectively. Metabolite III was not cyclized with weak nitrous acid to a triazolo[3,4-*a*]phthalazine in the same way as metab-

olite II. The peak obtained for III in the hydralazine assay was characterized by its retention time and spectral properties which were identical to those of IX.

The use of 4-methylhydralazine pyruvic acid hydrazone [12] as internal standard for the quantification of metabolite II rather than 4-methylhydralazine had no advantages in terms of accuracy or precision and resulted in considerably longer assay time due to the longer retention time of its derivative.

The use of excess strong acid (2 *N*) in the apparent hydralazine assay resulted in complete conversion of the hydrazones II—IV to the hydralazine derivative (IX) which was quantitated. This assay therefore provided an estimate of the hydralazine plus hydrazones level. If a smaller volume or more dilute acid was used relative to the plasma volume, some conversion of II to VII occurred and the total or apparent level was under-estimated.

The chromatographic and fluorimetric conditions for the assay of I, II and apparent hydralazine were identical and for the assay of metabolites V—VII, only a slight change in the mobile phase, aqueous phosphoric acid—acetonitrile from 85 : 15 to 80 : 20 was required for the chromatography. Using pre-programmed chromatographic conditions the injection and quantitation of large numbers of samples containing I, II and V—VII was carried out automatically overnight.

Specificity of the HPLC assays

Using 0.02 *N* hydrochloric acid, there was no interference by the hydralazine hydrazones (II and IV) in the hydralazine assay. The α-ketoglutaric acid hydrazone (III) was converted to the hydralazine derivative to the extent of 10%, however since plasma levels of this metabolite appear to be less than 10% of the apparent hydralazine level this represents an insignificant interference. The retention times of the other known fluorescent metabolites of hydralazine

TABLE IV

RETENTION TIMES (sec)

Derivative/metabolite	Mobile phase (1.5 m*M* phosphoric acid—acetonitrile)	
	85 : 15	80 : 20
Metabolite VI	230	160
3-Hydroxyquinidine	230	160
Metabolite V	310	230
4-Hydroxypropranolol	350	270
Tetrazolo[3,4-α]phthalazine (IX)*	400	–
N-Desisopropylpropranolol	430	310
Quinidine	430	310
Metabolite VII	490	320
Dihydroquinidine	530	350
6-Methyltetrazolo[3,4-α]phthalazine (X)**	600	–
Propranolol	920	620
3-Trifluoromethyl-s-triazolo[3,4-α]-phthalazine***	–	680
Desipramine	–	1450
Imipramine	–	1650

*Hydralazine derivative from nitrous acid treatment.

**4-Methylhydralazine derivative from nitrous acid treatment.

***Internal standard for the assay of metabolites V—VII.

and propranolol and quinidine and their metabolites in the hydralazine and hydralazine pyruvic acid hydrazone assays are shown in Table IV. Quinidine eluted between IX and VII and dihydroquinidine eluted between VII and X. If present in high levels, quinidine and its metabolites could therefore interfere in the assays for I and II. In the assay for the metabolites, 3-hydroxyquinidine had a retention time similar to VI and interfered in the determination of this metabolite. Imipramine and desipramine eluted after the internal standard in all assays.

Stability of derivatized samples to storage

As a result of the rapid disappearance of hydralazine in freshly drawn blood samples, derivatization had to be performed within 5 min of collection and the actual time elapsed noted. However, after nitrous acid derivatization and basification plasma samples could be stored for considerable lengths of time in polystyrene tubes at a variety of temperatures without a reduction in recovery (Table V).

TABLE V

STABILITY OF DERIVATIZED PLASMA SAMPLES TO STORAGE IN THE ASSAYS OF I AND II

The added amount was 5.0 μmole/l for I and II.

Storage conditions	Level obtained (μmole/l)	
	I	II
Immediate assay	5.1 ± 0.1*	5.1 ± 0.2*
24 h at 20°	5.2 ± 0.3*	5.0 ± 0.2*
4 h at 4°	5.1 ± 0.2*	5.0**
24 h at −20°	5.1 ± 0.1*	5.2 ± 0.5*
1 week at −20°	4.9**	5.1**
2 weeks at −20°	5.0**	5.3**

*Five determinations.
**Single determinations.

Reaction of I in plasma in vitro at 37°

Hydralazine (I) reacted rapidly with endogenous pyruvic acid in plasma at 37° to form II and as the level of I fell, there was a corresponding rise in the level of II (Fig. 4). The sum of the levels of I and II at any time was not significantly different from the apparent hydralazine level measured simultaneously and none of the metabolites V—VII were formed. Levels of I determined specifically at pH 5.5 declined to 0 μmole/l in 2 h, however estimations of the hydralazine level at pH 3.9 plateaued at 0.06 μmole/l probably due to interference by a small level of hydrazone III which was converted to IX. At sampling times up to 1 h there were no significant differences between the levels of I measured using the different acid strengths.

Pharmacokinetic studies

The plasma level time courses of I and its metabolites following separate oral and intravenous administration of the drug to a fasting healthy volunteer are shown in Fig. 5a and b respectively. The areas under the concentration—time

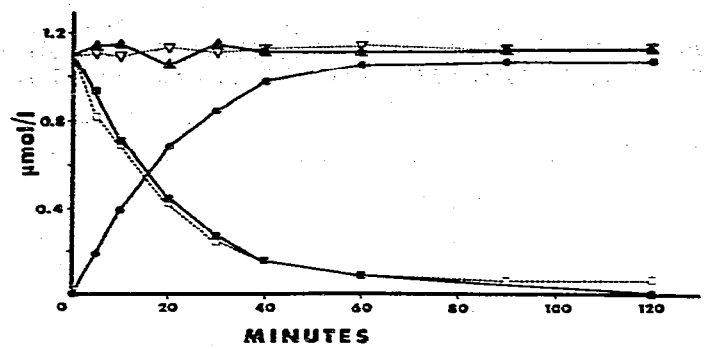


Fig. 4. Reaction of I (1.1 µmole/l) in fresh plasma at 37°. □——□ = I measured at pH 3.9; ■——■ = I measured at pH 5.5; ●——● = II; ▲——▲ = apparent hydralazine; ▽ ... ▽ = I + II.

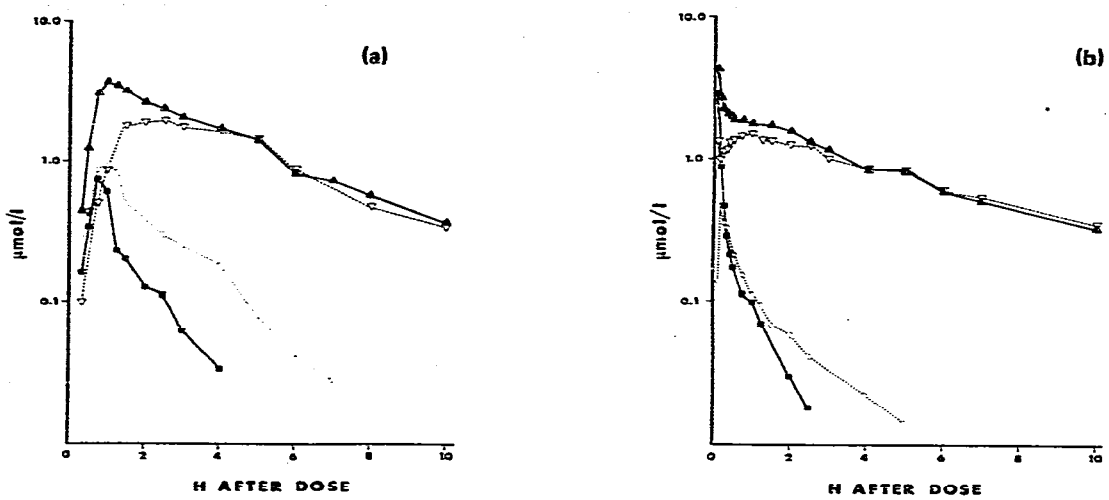


Fig. 5. Plasma level—time course of hydralazine and metabolites following an oral dose of hydralazine (1.0 mg/kg) to a healthy volunteer (slow acetylator) (a) and following an intravenous infusion (over 5 min) of hydralazine (0.375 mg/kg) to the same volunteer (b). ■——■ = I; ▽---▽ = II; ◇ ... ◇ = VII; ▲——▲ = apparent hydralazine.

curve (AUC) for each component are summarized in Table VI. The subject was a slow acetylator of sulfamethazine.

Following oral administration, the AUC for I constituted only 4.3% of the apparent hydralazine AUC. A very rapid disappearance of I in plasma was observed with no detectable level being found 5 h after the dose. The major component of the apparent hydralazine present was II which accounted for 100% of the apparent hydralazine at times beyond 4 or 5 h after the dose. The only triazolo[3,4-*a*]phthalazine metabolites observed were VI and VII with only traces of the former.

Following intravenous administration of hydralazine, the levels of I declined rapidly paralleling the in vitro loss of I at 37° in fresh plasma. Again the major component of the apparent hydralazine present was II and at times greater than 4 h after the dose constituted 100% of the apparent nydralazine level. Metab-

TABLE VI

AREAS UNDER CURVE (AUC) FOR HYDRALAZINE AND METABOLITES FOLLOWING ORAL (1 mg/kg) AND INTRAVENOUS ADMINISTRATION (0.375 mg/kg) TO A HEALTHY VOLUNTEER

Drug/metabolite	AUC (μmole/l/h)	
	Oral	Intravenous
Apparent hydralazine	17.8	11.51
I	0.77	0.54
II	11.30	9.96
VI	0.24	0
VII	1.87	0.65

olite VII was the only triazolophthalazine metabolite observed after intravenous administration of the drug. In both the oral and intravenous dose studies, the levels of metabolite II slowly increased and then declined with a half-life of 4.0 and 3.0 h respectively. In the intravenous study, the levels of apparent hydralazine did not decline in a simple mono- or bi-exponential fashion but showed fluctuations.

DISCUSSION

Selective and convenient HPLC assays for hydralazine (I), its pyruvate hydrazone (II) and metabolites (V—VII) in plasma were developed. I was quantitated using 4-methylhydralazine as internal standard after selective conversion to the respective tetrazolo[3,4-*a*]phthalazines (IX and X) using dilute nitrous acid at final pH 5.5. II was converted to VII in high yield with nitrous acid at pH 3.9 and was quantitated after subtraction of the endogenous plasma level of VII. The native fluorescence of tetrazolo[3,4-*a*]phthalazine (IX) and V—VII allowed the sensitive detection of very low plasma levels after oral and intravenous dosage. Other assay methods for I were investigated without success. For example, the addition of propionic anhydride to buffered plasma under a variety of conditions only partly converted I to 3-ethyl-*s*-triazolo[3,4-*a*]phthalazine (<25%).

For the quantification of apparent hydralazine levels, that is, hydralazine plus all hydrazones, strongly acidic conditions were employed. This procedure was similar to that published [4] with the exception that excess acid (relative to the plasma volume) was used to ensure that complete conversion of the hydrazones to hydralazine was accomplished. Measurement of the released hydralazine was carried out by conversion to IX followed by HPLC with fluorescence detection and using 4-methylhydralazine as the internal standard. Published methods [4, 13] have often under-estimated the apparent hydralazine level due to incomplete conversion of the hydrazones. For example, only 50% recovery of II was observed using the method described by Zak et al. [13], the remainder probably being converted to VII.

The experiment we reported in an earlier publication [12] in which I was added to fresh plasma and the levels of I, II and apparent hydralazine were measured, was repeated at 37° using the simplified methods described presently. The reaction was noticeably faster at 37° and after 15 min half the hydralazine was converted to II with no measurable level of hydralazine in plasma after 2 h.

The very rapid reaction of hydralazine with pyruvic acid in plasma indicates the necessity to derivatize samples immediately after drawing the blood. This was accomplished by preparing tubes containing the required amount of sodium nitrite, rapidly centrifuging blood (30 sec) and immediately adding the internal standard and acid. Derivatization occurred very rapidly trapping hydralazine as the stable tetrazolo[3,4-*a*]phthalazine derivative (IX) and II as derivative VII. Following basification, the samples were stable to storage for long periods.

In the case of the metabolites V, VI and VII and apparent hydralazine it was adequate to freeze the plasma samples immediately (−20°) and assay them the next day. No significant decrease in the level of apparent hydralazine occurred over 24 h at −20°. Previous observations that the apparent hydralazine level declines slowly even at −20° may be misleading due to the incomplete conversion of II using the methods published.

In the pharmacokinetic studies hydralazine constituted only a small fraction of the AUC of apparent hydralazine with hydrazone II accounting for all the apparent hydralazine 4 h after the oral and intravenous doses. This result is consistent with our earlier observation that more than 90% of the apparent hydralazine in plasma from patients taking oral hydralazine at steady-state is present as the pyruvate hydrazone. At times immediately following the doses there was a fraction of the apparent hydralazine which was not hydralazine or its pyruvate hydrazone. Using the combined levels of I and 70% of III obtained at pH 3.9 a significant fraction of this material was estimated to be the *α*-ketoglutaric acid hydrazone III.

Acetylation has been described as the major route of hydralazine elimination [5] and it is therefore important in pharmacokinetic studies to measure metabolites V—VII all of which are derived from acetylation. Wagner et al. [18] have reported that the major urinary metabolite of hydralazine in man is conjugated VI. Only small levels of unconjugated metabolite VI were observed in plasma and attempts to measure levels of conjugated material were seriously hampered by interference from relatively large amounts of fluorescence material present in several different commercial preparations of the mixed glucuronidase—sulphatase enzymes [19].

Recently published work [20, 21] has confirmed our observation [11] that the pyruvic acid hydrazone of hydralazine is inactive when administered intravenously to animals, however, the activities of this metabolite and the other hydralazine metabolites still await investigation in man.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. W. Riess, Research Department, Pharmaceuticals Division, Ciba-Geigy, Basle, Switzerland, for generously donating the internal standard, 4-methyl-1-hydrazinophthalazine. The authors also wish to thank Dr. D. Johnson of the Dept. of Endocrinology and Gynaecology, The Queen Elizabeth Hospital for assistance with the gas—liquid chromatography—mass spectrometry.

REFERENCES

1 J. Koch-Weser, Amer. Heart J., 95 (1978) 1.
2 A.R. Schulert, Arch. Int. Pharmacodyn., 132 (1961) 1.
3 J.M. Lesser, Z.H. Israili, E. Davis and F.G. Dayton, Clin. Pharmacol. Ther., 14 (1973) 140.
4 D.B. Jack, S. Brechbühler, P.H. Degen, P. Zbinden and W. Riess, J. Chromatogr., 115 (1975) 87.
5 M.M. Riedenberg, D. Drayer, A.L. DeMarco and C.T. Bello, Clin. Pharmacol. Ther., 14 (1973) 970.
6 T. Talseth, Eur. J. Clin. Pharmacol., 10 (1976) 183.
7 T. Talseth, Eur. J. Clin. Pharmacol., 10 (1976) 311.
8 T. Talseth, Eur. J. Clin. Pharmacol., 10 (1976) 395.
9 T. Talseth, Clin. Pharmacol. Ther., 21 (1976) 715.
10 A. Melander, K. Danielson, A. Hanson, B. Rudell, B. Schersten, T. Thulin and E. Wahlin, Clin. Pharmacol. Ther., 22 (1977) 104.
11 P.A. Reece, P.E. Stanley and R. Zacest, J. Clin. Exp. Pharmacol. Physiol., 5 (1978) 248.
12 P.A. Reece, P.E. Stanley and R. Zacest, J. Pharm. Sci., 67 (1978) 1150.
13 S.B. Zak, G. Lukas and T.G. Gilleran, Drug Metab. Dispos., 5 (1977) 116.
14 P.H. Degen, J. Chromatogr., 176 (1979) 375.
15 P.A. Reece and R. Zacest, J. Clin. Exp. Pharmacol. Physiol., 6 (1979) 207.
16 K.D. Haegele, H.B. Skrdlant, N.W. Robie, D. Lalka and J.K. McNay, Jr., J. Chromatogr., 126 (1978) 517.
17 T. Talseth and K.H. Landmark, Eur. J. Clin. Pharmacol., 11 (1977) 33.
18 J. Wagner, J.W. Faigle, P. Imhof and G. Liehr, Arzneim.-Forsch., 27 (1977) 2388.
19 M. Lo, P.A. Peece and S. Riegelman, J. Clin. Exp. Pharmacol. Physiol., 6 (1979) in press.
20 W.A. Clementi, H.B. Skrdlant, J.L. McNay and K.D. Haegele, Pharmacologist, 20 (1978) 190.
21 A.M.M. Shepherd, W.A. Clementi, K.D. Haegele, T.M. Ludden, J.L. McNay, H.B. Skrdlant and T. Talseth, Clin. Res., 26 (1978) A782.