ammonium part of the molecules by acid hydrolysis, indicate that the presence of a relatively nonpolar lipophilic part in the molecule of organic cations is a prerequisite for efficient biliary excretion.

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Interference in Assays for Hydralazine in Humans by a Major Plasma Metabolite, Hydralazine Pyruvic Acid Hydrazone

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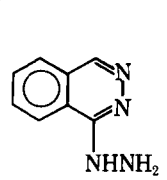
Abstract □ The present study showed that published spectrophotometric and GLC methods for hydralazine in plasma do not distinguish between the drug and a major plasma metabolite, hydralazine pyruvic acid hydrazone. These methods involve the acidic treatment of the sample, which hydrolyzes the hydrazone back to hydralazine. A specific GLC assay for the hydrazone was developed and involves its selective extraction from plasma and transformation to 3-trifluoromethyl-*s*-triazolo[3,4-*a*]phthalazine. This derivative could be sensitively measured by GLC using an electron-capture detector. With this procedure, it was shown that most "apparent hydralazine" in plasma is the hydrazone, which forms rapidly from hydralazine and endogenous pyruvic acid. Previous work indicated that the hydrazone was inactive when administered intravenously to rabbits.

Keyphrases □ Hydralazine—interference in published GLC and spectrophotometric analyses in plasma by major metabolite □ Metabolites—hydralazine pyruvic acid hydrazone, GLC analysis in plasma □ GLC—analysis, hydralazine pyruvic acid hydrazone in plasma □ Antihypertensives—hydralazine, interference in published GLC and spectrophotometric analyses in plasma by major metabolite

The vasodilator hydralazine, 1-hydrazinophthalazine (I), is an effective agent for the treatment of arterial hypertension (1). It undergoes extensive metabolism in humans and animals, and Metabolites II–VIII were identified (1–11). Acetylation was proposed as the major pathway for hydralazine clearance (2–8), where the acetylated drug, 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (III), may be further metabolized to 3-hydroxymethyl-*s*-triazolo[3,4-*a*]-phthalazine (IV) and its glucuronide conjugate (2, 11).

Although hydralazine can form hydrazones readily with compounds having an active carbonyl group, hydralazine pyruvic acid hydrazone, 1-hydrazinophthalazine pyruvic acid hydrazone (II), was identified only as a minor metabolite in rat urine following oral administration of hydralazine (2, 4). Fresh human blood contains pyruvic acid in a concentration of approximately 100 μmoles/liter, which is a considerable excess to the commonly found therapeutic levels of "apparent hydralazine" and appears to favor formation of II. Preliminary findings indicated (12) that plasma apparent hydralazine, or that measured



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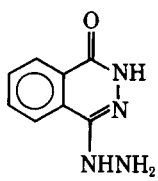

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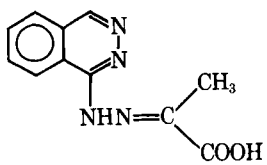

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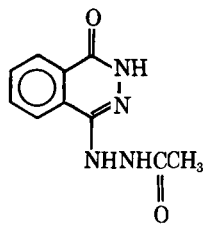

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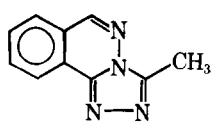

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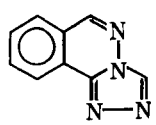

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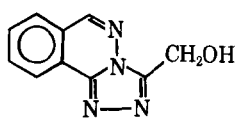

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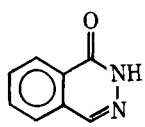

VIII

0022-3549/78/0800-1150$01.00/0

$$\text{II} \xrightarrow[H_2O]{\text{acid}} \text{I} + CH_3\overset{\overset{O}{\|}}{C}COOH$$

Scheme I

by the published spectrophotometric (13) and GLC (14) assays, was composed largely of II.

In the present study, methods for plasma hydralazine were examined for specificity using synthesized hydralazine metabolites, and investigations of the significance of plasma II levels were undertaken. Formation of II from I and pyruvic acid in plasma and water and the stability of II in various buffers also were studied.

EXPERIMENTAL

Syntheses—The hydralazine metabolites (II–VIII) were synthesized by published methods (2–11, 15), except 4-hydrazinophthalazin-1(2*H*)-one (V), which was synthesized by refluxing a solution of 1,4-dihydrazinophthalazine sulfate[1] (2.7 g) in water (100 ml) for 30 min. The mixture was concentrated under reduced pressure and cooled to precipitate the desired product (272 mg, 12.5%), mp 155–156°; mass spectroscopy: found *m/e* 176, $C_8H_8N_4O$ requires *m/e* 176 (M^+).

Anal.—Calc. for $C_8H_8N_4O{\cdot}0.5H_2O$: C, 51.9; H, 4.9; N, 30.3. Found: C, 51.9; H, 5.0; N, 30.7.

3-Trifluoromethyl-*s*-triazolo[3,4-*a*]phthalazine (IX) was synthesized by the method of Lesser *et al.* (3).

Apparent Hydralazine Assays—The spectrophotometric hydralazine method of Schulert (13), as modified by Zacest and Koch-Weser (6), was used. The GLC assay of Jack *et al.* (14) was employed without modification; 0.5 ml of plasma was used, and the final residue, after evaporation of the benzene, was dissolved in toluene (1 ml). Replicate samples (1 μl) were injected into the gas–liquid chromatograph[2] with an automatic sample injector[3].

Both chromatographs were equipped with ^{63}Ni-electron-capture detectors. A comparison of the two methods was undertaken by assaying duplicate plasma samples from 94 hypertensive patients receiving chronic oral hydralazine therapy.

Acid Lability of II—At a concentration of 43 μmoles/liter, II was added to Clark–Lubs potassium chloride buffer for pH 1.0 and 2.0; to Clark–Lubs potassium hydrogen phthalate buffer for pH 2.5, 3.0, and 4.0; to Clark–Lubs sodium borate buffer for pH 7.0, 8.0, 9.0, and 10.0; and to aqueous 1 *N* HCl for pH 0.1. The rate and extent of decomposition of II (Scheme I) were determined by UV spectrophotometry at 21° from the absorbance of the peak with $\lambda_{max} \simeq 340$ nm ($\epsilon_{max} \simeq 1.0 \times 10^4$) (Fig. 1). Hydralazine had insignificant absorption at this wavelength. The UV spectrum and melting point of the decomposition product were compared

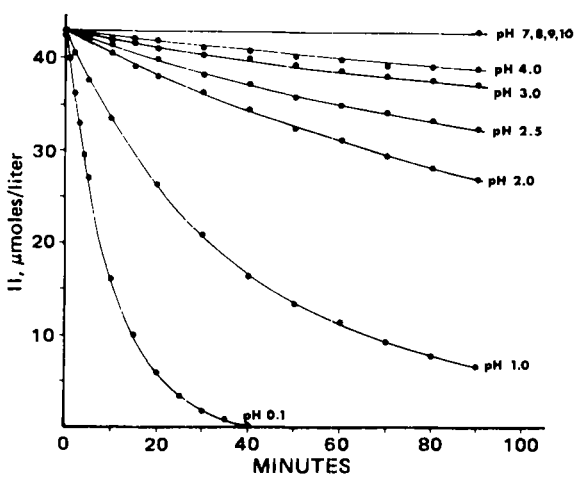

Figure 1—*Stability of II determined by UV spectrophotometry at* $\lambda_{max} \simeq 340$ *nm* ($\epsilon \simeq 1.0 \times 10^4$) *and 21°.*

[1] Ciba-Geigy, Basel, Switzerland.
[2] Packard model 419 or a Pye Series 104.
[3] Packard model 700 or a Pye model S4.

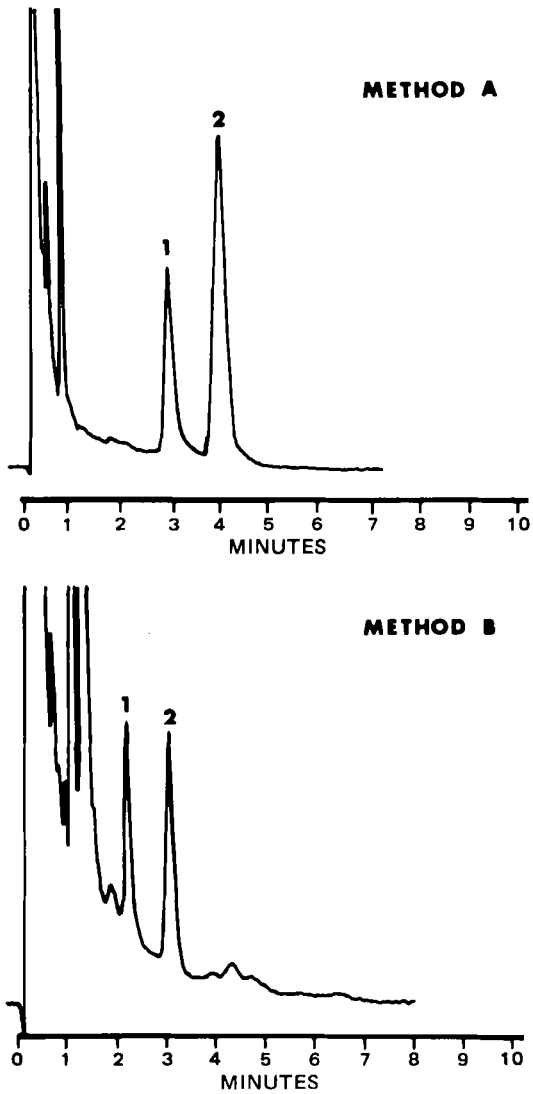

Figure 2—*GLC traces for II assayed by Methods A and B. Key: Peak 1, II; and Peak 2, X (internal standard).*

with those of hydralazine.

Interference of Hydralazine Metabolites in Apparent Hydralazine Assays—Compounds II–V, VII, and VIII were added to separate fresh plasma samples to give a final concentration of 7.5 μmoles/liter and assayed for apparent hydralazine by the spectrophotometric and GLC procedures.

Hydralazine Pyruvic Acid Hydrazone (II) Assays—*Method A*—To 1 ml of plasma, obtained by rapid centrifugation of the blood sample at 4°, was added the internal standard, 4-methylhydralazine pyruvic acid hydrazone (X) (7.5 μmoles/liter). Compound X was synthesized from 4-methylhydralazine[2] and pyruvic acid by the method described for II. Aqueous 4 *N* HCl (45 μl), sodium sulfate (500 mg, pH 3.0), and chloroform (10 ml) were then added. The extraction was performed within 30 sec of acidification by shaking the mixture at 200 rpm for 10 min.

The chloroform layer was then poured into a conical centrifuge tube (the semisolid aqueous mixture adhered to the side of the extraction tube) and evaporated rapidly under a stream of nitrogen at 45°. The residue was treated with trifluoroacetic anhydride (100 μl) at 21° for 5 min. The excess trifluoroacetic anhydride was evaporated under a stream of clean, dry nitrogen. The residue was then dissolved in toluene (1 ml), and 1 μl was injected onto the gas–liquid chromatograph[2] equipped with a ^{63}Ni-electron-capture detector and a 1-m × 2-mm glass column packed with 3% OV-17 on Gas Chrom Q (Fig. 2).

The column flow rate was 50 ml/min (oxygen-free nitrogen), the makeup flow rate was 10 ml/min (nitrogen), the column temperature was 190°, the injector temperature was 220°, and the detector temperature was 300°. The recovery of II (7.5 μmoles/liter) added to plasma and assayed by this method was determined in replicate by comparison with

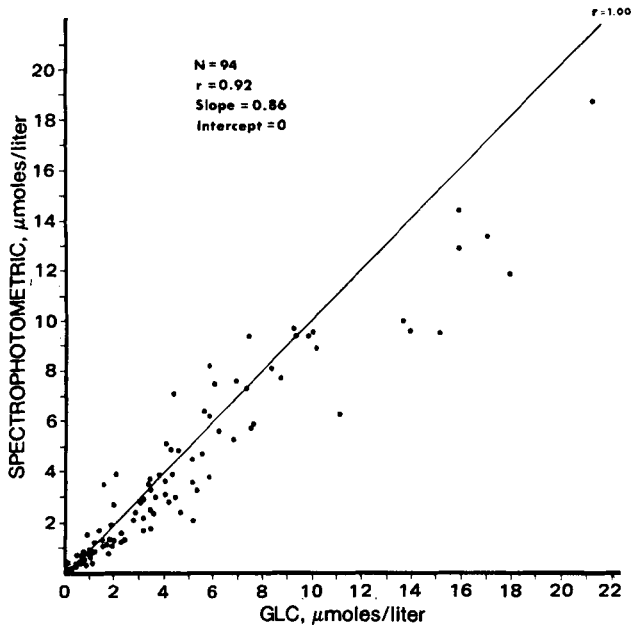

Figure 3—*Comparison of the GLC and spectrophotometric assays for apparent hydralazine in 94 hypertensive patients receiving chronic oral hydralazine therapy.*

the peak obtained for 100 pg of IX injected into the gas chromatograph.

A sample of II was derivatized as described, dissolved in methanol, and injected into a gas chromatograph–mass spectrometer[4]. The total ion current and molecular ion (*m/e* 252) were then monitored. The mass spectrum of the single peak was compared with that of an authentic sample of IX.

Method B—An alternative to Method A was to chromatograph II and the internal standard without prior derivatization. The residue (see *Method A*) was dissolved in methanol (50 μl), and 1 μl was injected into the gas–liquid chromatograph equipped with a 1-m × 2-mm glass column packed with 3% OV-17 on Gas Chrom Q and a nitrogen detector[5]. The flow rates were: nitrogen carrier gas, 35 ml/min; hydrogen, 30 ml/min; and air, 150 ml/min. The temperatures were: column, 245°; injector, 265°; and detector, 270° (Fig. 2).

To check for interference, hydralazine (7.5 μmoles/liter) and its metabolites were added to plasma and samples were assayed for II by Methods A and B.

Formation of II in Plasma and Water—Hydralazine was added to fresh heparinized plasma to give a final concentration of 7.5 μmoles/liter and maintained at 21°. Samples (1 ml) were taken over 4 hr and assayed for II by Method A. Samples were also assayed for apparent hydralazine using the GLC method (14) and for pyruvic acid using a commercially available test kit[6].

The reaction of hydralazine (78 μmoles/liter) with pyruvic acid (12.5 mmoles/liter) in water was monitored by UV spectrophotometry at 21°. The absorbance at $\lambda_{max} \simeq 340$ nm was used to determine the concentration of II in the mixture.

Comparison of Apparent Plasma Hydralazine and II Levels—Samples of plasma from 37 hypertensive patients receiving chronic oral therapy with hydralazine and at steady state (drawn >2 hr after the last dose) were each assayed for II (Methods A and B) and for apparent hydralazine using the spectrometric and/or GLC hydralazine method. The relationship of the concentrations of the two compounds in individual samples was thus obtained.

RESULTS

Comparison of Apparent Hydralazine Assays—A comparison of the spectrophotometric and GLC plasma assays for apparent hydralazine in 94 hypertensive patients receiving chronic oral hydralazine showed that the results from the two methods correlated satisfactorily ($r = 0.92$) (Fig. 3). The line of best fit determined by the method of least squares had a slope of 0.86 and a zero intercept.

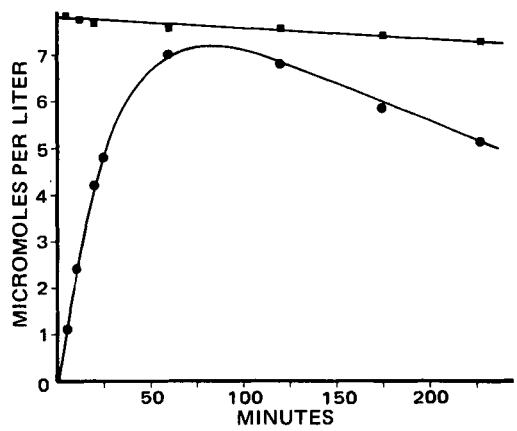

Figure 4—*Formation of II (●) from hydralazine added to fresh plasma containing endogenous pyruvic acid (68 μmoles/liter), and the stability of apparent hydralazine (■) in the same plasma measured by the GLC method of Jack* et al. *(14).*

Acid Lability of II—Compound II, added to plasma to give a concentration of 7.5 μmoles/liter and assayed by the spectrophotometric and GLC methods, gave an apparent hydralazine plasma level of 7.5 μmoles/liter. The other hydralazine metabolites did not interfere. Compound II was converted rapidly to hydralazine at low pH, and hydrolysis in aqueous 1 *N* HCl was complete in 40 min at 21° (Fig. 1). In buffer at pH 3.0, II (43.0 μmoles/liter) decomposed at a rate of 60 nmoles/liter/min. The UV spectrum and melting point of the decomposition product of II were identical with those of hydralazine. During plasma treatment with nitrous acid in the GLC hydralazine assay, the pH of the solution was less than 1 (for 15 min). Similarly, the pH required for the formation of the *p*-hydroxybenzaldehyde derivative in the spectrophotometric assay was 1.5 (70° for 10 min).

II Assays—Treatment of II and its 4-methyl analog with trifluoroacetic anhydride in Method A gave the respective trifluoromethyl-*s*-triazolo[3,4-*a*]phthalazines with GLC retention times of 2.9 and 4.0 min. This conversion was confirmed for II by GLC–mass spectrometry. The mass spectrum of the single product was identical with that of an authentic sample of IX.

Standard curves obtained using Methods A and B for a series of plasma standards were linear over the range of 0.1–10.0 μmoles of II/liter. The coefficient of variation for the 3.0-μmole/liter plasma standard assayed in replicate was 2.0% for Method A and 5.5% for Method B. The mean recovery of II added to plasma (7.5 μmoles/liter) and determined using Method A was 6.9 μmoles/liter (*SD* ±0.1 μmole/liter). Hydralazine was not extracted from the acidified plasma in Methods A and B, and neither it nor the hydralazine metabolites interfered in the assays for II.

Formation of II—Compound II was formed rapidly (Fig. 4) following the addition of hydralazine to fresh human plasma containing endogenous pyruvic acid (68 μmoles/liter). The second-order rate constant for this reaction was 7.4 liters/mole/sec (21°). There was a slight fall (1.5%/hr) in the apparent hydralazine level measured over the same period. The level of II reached a peak and then slowly declined.

The second-order rate constant for the reaction of hydralazine with pyruvic acid in water was 1.9 liters/mole/sec.

Comparison of Apparent Plasma Hydralazine and II Levels—There was good correlation between apparent hydralazine and plasma levels of II ($r = 0.94$, $p = 0.00001$) (Fig. 5). The slope of the line of best fit determined by the method of least squares was 0.94 ± 0.05, and the intercept was not significantly different from zero.

DISCUSSION

The published apparent hydralazine assays employing GLC and UV spectrophotometry correlated well, but both lacked specificity for unmetabolized hydralazine. The interfering metabolite was II, which was unstable at low pH and completely converted to free hydralazine under the conditions of sample treatment (Scheme I).

The recovery of II added to plasma in Method A was high (92%), and decomposition of II at the pH used in the extraction step (pH 3.0) was sufficiently slow (0.15%/min) that it did not interfere in the assay. The derivative obtained in Method A was highly electron capturing, provided

[4] Model AEI MS-30.
[5] Packard model 713.
[6] Boehringer diagnostic kit.

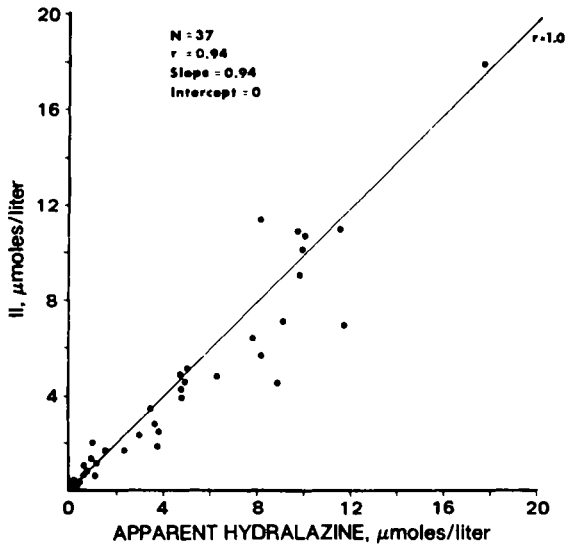

Figure 5—*Comparison of apparent hydralazine (measured by the spectrophotometric and/or GLC hydralazine methods) and II levels at steady state in plasma samples drawn from hypertensive patients receiving chronic oral hydralazine therapy.*

good sensitivity (10 ng/ml), and gave a linear response over the concentration range used. Estimates of the plasma II levels in patients receiving oral hydralazine indicated that more than 90% of the apparent hydralazine measured by the published assays was present as II. These findings are consistent with those of Zak *et al.* (16) who found significantly high levels of "acid-hydrolyzable conjugates" in the plasma of human subjects after oral administration of hydralazine. The discrepancy between II and apparent hydralazine levels in individual samples may be due to other acid-labile hydrazones or free hydralazine itself.

When hydralazine was added to fresh heparinized human plasma containing endogenous pyruvic acid or water containing added pyruvic acid, II formed rapidly. The second-order rate constants (17) for the reaction in each of these systems were 7.4 and 1.9 liters/mole/sec, respectively. The formation rate of II *in vivo* can be expected to be even higher because of the continual replenishment of consumed pyruvic acid and the higher physiological temperature (37°). Therefore, any hydralazine entering the circulation probably would be rapidly metabolized to II and circulate as such. The relative concentrations of pyruvic acid, I, and II in human plasma immediately following administration of the drug by oral and intravenous routes are currently being evaluated.

At present, there is no satisfactory explanation for the slow decline in the level of II (Fig. 4) following its formation from hydralazine. However, the slow decomposition of apparent hydralazine in plasma was observed previously (3, 13, 18).

In parallel with the current findings with hydralazine, Dauphinee *et al.* (17) showed that isoniazid also reacts rapidly with pyruvic acid in water. Comparison with the present work indicated that the rate of this reaction was approximately 100 times slower than that of hydralazine with pyruvic acid in water. This result reflects the lower reactivity of the hydrazide group of isoniazid compared with that of the hydrazino group of hydralazine. Even so, 25% of the total isoniazid in a human subject's plasma was present as unidentified hydrazone 60 min after an oral dose of isoniazid (18).

Compound II did not show any hypotensive activity when administered intravenously to rabbits (12). The lack of hypotensive activity of the metabolite in the rabbit suggests that it may also lack activity in humans.

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