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High-Performance Liquid Chromatographic Studies of Reaction of Hydralazine with Biogenic Aldehydes and Ketones

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Abstract □ To understand hydrazone formation in hydralazine metabolism, the reaction of hydralazine with various biogenic aldehydes and ketones (acetone, pyruvic acid, acetoacetic acid, formaldehyde, and acetaldehyde) in pH 7.4 buffer was studied for potential alterations in hydralazine pharmacokinetics secondary to alcoholism and diabetes. The corresponding hydrazones were isolated, and their structures were characterized. High-performance liquid chromatography was used to monitor the reactions. An aqueous solvent reversed-phase liquid chromatographic system was used to separate hydralazine and its derivatives. Reaction of hydralazine with formaldehyde or acetaldehyde produced the corresponding hydrazones. Formation of an *s*-triazolo ring system yielded the known *s*-triazolo[3,4-α]phthalazine and 3-methyl-*s*-triazolo[3,4-α]phthalazine metabolites, which also were isolated and characterized and suggested nonenzymatic metabolism.

Keyphrases □ Hydralazine—reaction with biogenic aldehydes and ketones, hydrazone formation, high-performance liquid chromatographic analysis □ Antihypertensive agents—hydralazine, reaction with biogenic aldehydes and ketones, hydrazone formation, high-performance liquid chromatographic analysis □ High-performance liquid chromatography—analysis, hydralazine reaction with biogenic aldehydes and ketones, hydrazone formation

The mechanism of hydralazine condensation with biogenic aldehydes and ketones is significant. The reaction, involving hydrazone formation, may be enhanced in alcoholism, diabetes, and fasting states and during prolonged hydralazine administration. Hydrazone formation is important for investigating hydralazine toxicity and alterations in hydralazine metabolism and pharmacokinetics. While hydrazone formation has been evaluated *in vivo* (1–5), no supporting chemical studies have been reported.

A recently developed high-performance liquid chromatographic (HPLC) method (6) is sufficiently sensitive for clinical assay of derivatized hydralazine. The reaction of hydralazine with biogenic aldehydes and ketones apparently is nonenzymatic. The chemistry in an *in vitro* homogeneous solution at physiological conditions should be analogous to the *in vivo* process. Thorough study of this chemistry is complicated by the numerous biogenic ketones and aldehydes available to undergo this condensation *in vivo*.

This report describes the use of HPLC to determine the overall reaction of hydralazine with biogenic aldehydes and ketones to form hydrazones and the subsequent reaction of specific hydrazones to form *s*-triazolo[3,4-α]phthalazine and 3-methyl-*s*-triazolo[3,4-α]phthalazine. HPLC is well suited to this objective since the reversible reaction is usually sufficiently slow to permit discrete analysis of the reaction mixtures. This investigation was undertaken as a part of a long-term study of *in vivo* hydrazone formation from hydralazine in drug toxicity.

EXPERIMENTAL

Materials—Hydralazine[1], acetaldehyde[2], pyruvic acid[2], formaldehyde[2], 8-chlorotheophylline[3], acetone[4], and acetoacetic acid[4] were used as obtained. High purity samples of *s*-triazolo[3,4-α]phthalazine (III*a*) and 3-methyl-*s*-triazolo[3,4-α]phthalazine (III*b*) were prepared (5, 7) and used as standards.

Apparatus—A high-pressure liquid chromatograph[5] was equipped with a septumless injector port[6] and variable-wavelength UV absorption[7] and fluorescence detectors[8]. The column eluate was monitored by UV absorption at 240 nm and by fluorometry with excitation at 240 nm and

[1] Courtesy of Dr. M. Wilhem, Ciba-Geigy, Summit, N.J.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Fisher Scientific Co., Pittsburgh, Pa.
[5] Model 6000A, Waters Associates, Milford, Mass.
[6] Model U6K, Waters Associates, Milford, Mass.
[7] Model SF-770, Schoeffel Instruments Corp., Westwood, N.J.
[8] Model FS-970, Schoeffel Instruments Corp., Westwood, N.J.

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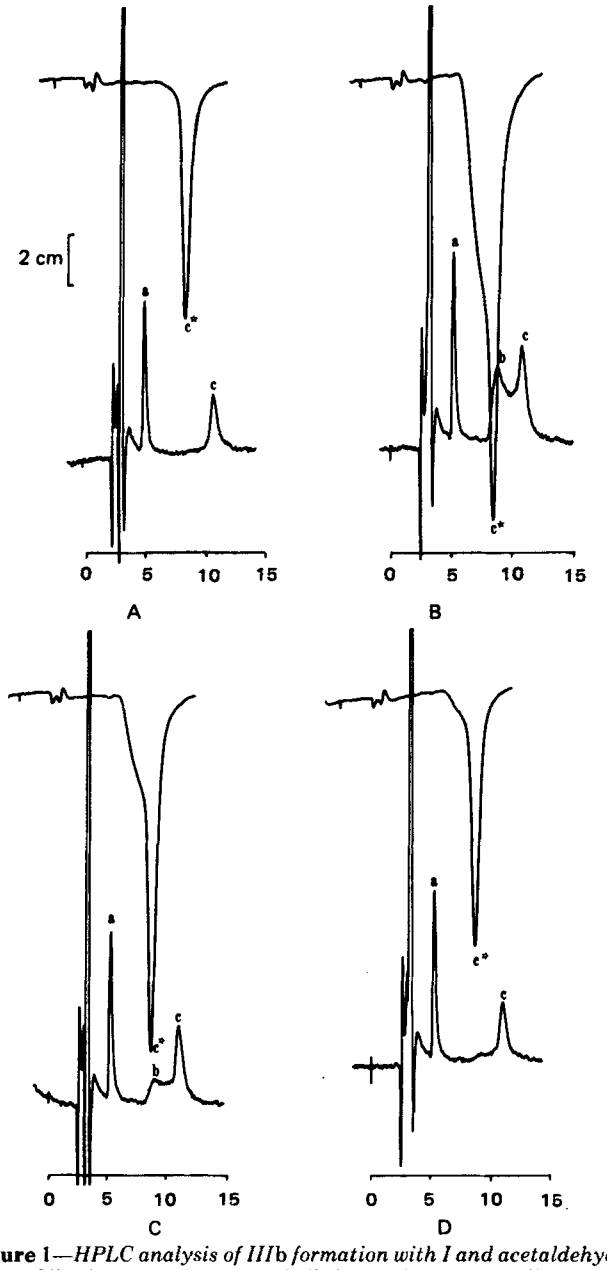


Figure 1—*HPLC analysis of IIIb formation with I and acetaldehyde. The mobile phase was 23% acetonitrile in 0.01* M *acetate buffer, pH 4.0, with a 1.0-ml/min flow rate. Key: A, standard IIIb, 1 μg/ml; B, mixture of 1 μg of I/ml with acetaldehyde after 5 min of mixing; C, Sample B after 30 min; and D, Sample B after 45 min. The chromatograms consist of both the UV (bottom) and fluorescence (top) detections. Peak a represents 8-chlorotheophylline (internal standard); peak b is the intermediate hydrazone IVb; and c and c* are the UV and fluorescence peaks of IIIb, respectively.*

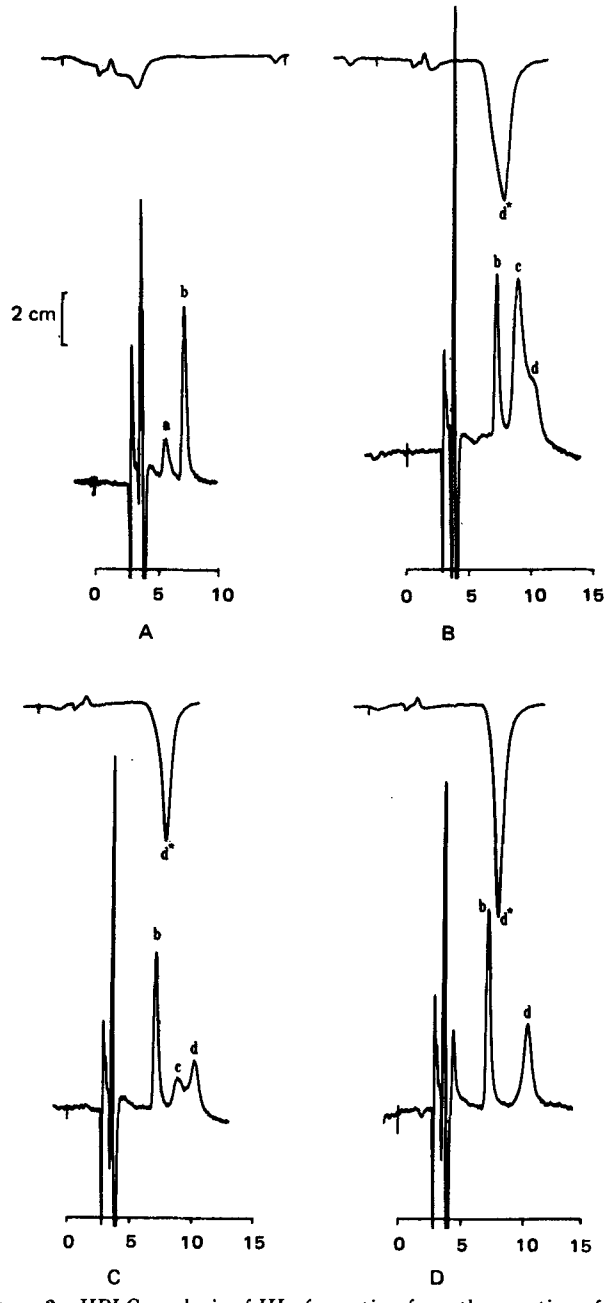


Figure 2—*HPLC analysis of IIIa formation from the reaction of hydralazine with formaldehyde. The mobile phase was 17% acetonitrile in 0.01* M *acetate buffer, pH 4.0, with a 1.0-ml/min flow rate. Key: A, standard I, 1 μg/ml; B, mixture of 1 μg of I/ml with formaldehyde after 5 min of mixing; C, Sample B after 30 min; and D, Sample B after 45 min. The chromatograms consist of both the UV (bottom) and fluorescence (top) detections. Peak a represents I; peak b is 8-chlorotheophylline (internal standard); peak c is the intermediate hydrazone IVa; and d and d* are the UV and fluorescence peaks of IIIa, respectively.*

emission above 389 nm. The UV detector was operated at 0.01 aufs with the fluorescence detector at 0.05 μamp full scale.

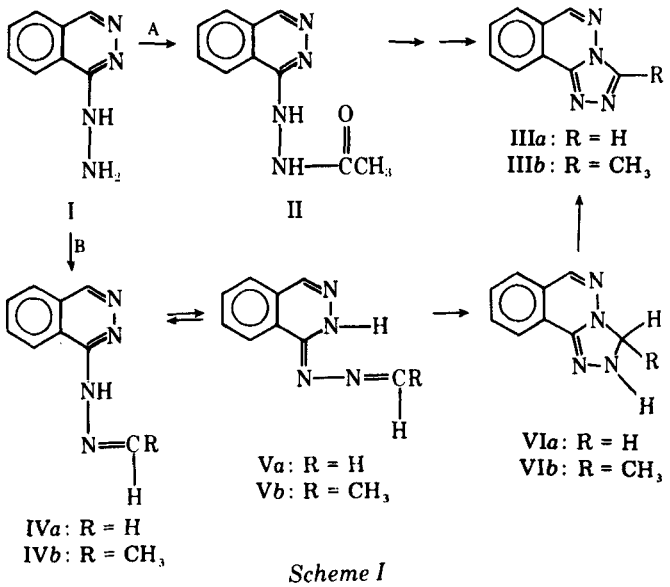
Chromatographic Parameters—Unless otherwise mentioned, the mobile phase was 17% acetonitrile[9] in 0.01 *M* sodium acetate, adjusted to pH 4.0 with acetic acid. It was pumped at 1.0 ml/min (1500 psi) through a stainless steel column (4 mm i.d. × 30 cm) with a high efficiency bonded-phase adsorption packing[10].

Procedure—The ketone or aldehyde was added to a 4-ml solution of hydralazine hydrochloride (1 μg/ml, 0.05 μ*M*) in 0.1 *M* phosphate buffer, pH 7.4, at 30°. The final concentration of the ketones or aldehydes was

[9] Nanograde, Mallinckrodt, St. Louis, Mo.
[10] μBondapak C_{18}, Waters Associates, Milford, Mass.

Table I—Retention Times of Hydralazine Derivatives with Aldehydes and Ketones Measured at a 1-ml/min Flow Rate with a Mobile Phase of 17% Acetonitrile in Acetate Buffer (pH 4)

Compound	Minutes
1-Hydrazinophthalazine (I)	5.7
1-Hydrazinophthalazine pyruvic acid hydrazone	4.8
1-Hydrazinophthalazine acetoacetic acid hydrazone	5.2
s-Triazolo[3,4-α]phthalazine (IIIa)	10.5
3-Methyl-*s*-triazolo[3,4-α]phthalazine (IIIb)	18.2
1-Hydrazinophthalazine acetone hydrazone	21.0



Scheme I

10 μg/ml. After 30 min, 6 μl of the reaction mixture was injected into the HPLC system. The hydrazones from hydralazine and other derived products were identified by comparison to standards of the acetone hydrazone, pyruvic acid hydrazone, and acetoacetic hydrazone of hydralazine prepared by literature procedures (5, 7) and to III*a* and III*b*.

Chemistry[11]—*1-Hydrazinophthalazine Acetaldehyde Hydrazone* (*IV*b)—Acetaldehyde (0.3 g, 6.82 mmoles) was added with stirring to 500 ml of 0.05 *M* phosphate buffer at pH 7.4 and 0.5 g (2.54 mmoles) of 1-hydrazinophthalazine (I) at 37°. The reaction mixture was stirred at 37° for 10 min and then filtered. The filtrate was dried *in vacuo* to yield a solid residue. Recrystallization from chloroform–ether gave 0.440 g (92%) of IV*b* as off-white crystals, mp 108–110° [lit. (7) mp 108–110°].

The 1-hydrazinophthalazine formaldehyde hydrazone (IV*a*) was prepared in the same manner as IV*b* using formaldehyde instead of acetaldehyde.

3-Methyl-s-triazolo[3,4-α]phthalazine (*III*b)—Acetaldehyde (0.3 g, 6.82 mmoles) was added with stirring to 500 ml of 0.05 *M* phosphate buffer at pH 7.4 and 0.5 g (2.54 mmoles) of I at 37°. The reaction mixture was stirred at 37° for 12 hr and then extracted with 3 × 100 ml of chloroform. The organic phase was dried over anhydrous magnesium sulfate, and the volatiles were removed under reduced pressure to give a solid residue. Recrystallization from chloroform–ether gave 0.388 g (85.3%) of III*b* as off-white crystals, mp 170–171° [lit. (7) mp 170–171°].

s-Triazolo[3,4-α]phthalazine (III*a*) was prepared in the same manner as III*b* using formaldehyde instead of acetaldehyde.

RESULTS AND DISCUSSION

Hydralazine was reported to condense *in vivo* with ketones to form hydrazones (2, 4, 5). While the details are not well understood, the first step was to assess the effect of the individual aldehydes and ketones on the overall formation of different hydrazones with hydralazine.

The formation of hydrazone derivatives with hydralazine and cyclized products was observed, and these compounds were separated using reversed-phase liquid chromatography. The retention times are listed in Table I. The linewidths of these peaks were within 1 min. The ketone hydrazones of hydralazine did not fluoresce under the conditions employed and were detected by UV. On elution, the more polar acetoacetic acid and pyruvic acid hydrazones preceded hydralazine. The less polar acetone hydrazone had a longer retention time. The chromatogram of the acetoacetic acid hydrazone also showed the acetone hydrazone, which occurs from the facile decarboxylation of acetoacetic acid.

The retention times listed in Table I may vary with changes in the percent of acetonitrile in the solvent, but the elution sequence remains the same. The reactions were supported by synthesis of the hydrazone derivatives at physiological conditions. In all instances, a reaction time of 30 min gave reactions with recrystallized yields of >80%.

The literature contains numerous examples of the *in vivo* formation of III*b*, a known major metabolite of hydralazine (8, 9), *via* enzymatic acetylation of hydralazine and subsequent dehydration (Scheme I, Pathway A) (3, 4). Hydralazine reaction with formaldehyde and acetaldehyde for 30 min at 37° resulted in the formation of III*a* and III*b*, respectively, which showed strong fluorescence and UV absorption. Figure 1 shows the chromatograms of the reaction mixture obtained during III*b* formation using a mobile phase of 23% acetonitrile and a 1.0-ml/min flow rate. The intermediate hydrazone product was observed prior to III*b* formation. Similar reaction intermediates also were found (Fig. 2) in the reaction of hydralazine with formaldehyde.

The results suggest an alternative competitive chemical pathway, B (Scheme I). This reaction occurs readily *in vitro* at 37° at pH 7.4 and involves the formation of the hydrazones IV*a* and IV*b*, which was supported by comparison of the retention times to the known hydrazone standards. The intermediate hydrazones IV*a* and IV*b* were observed (Figs. 1 and 2) and were isolated at simulated physiological conditions. Cyclization and subsequent oxidation of the 2,3-dihydro-3-methyl-*s*-triazolo[3,4-α]phthalazine (VI*b*) to III*b* occurs *via* the driving force of the ring system to become a completely aromatic system. Numerous analogous heterocyclic systems undergo similar oxidation (7, 10). Hydralazine reacts *in vitro* at 37°, pH 7.4, with formaldehyde to form III*a* *via* an analogous mechanism.

This HPLC approach is applicable to numerous related problems, in which hydrazines may react with biogenic aldehydes and ketones, because of its excellent selectivity and sensitivity (routine detection limits of 5 ng/ml). The pharmacokinetic importance of this reaction of hydralazine remains to be determined. *In vivo* experiments are planned.

[11] Melting points were taken on a Thomas–Hoover capillary apparatus and are uncorrected. The structures were confirmed by comparison of their IR (Beckman model 18A) and NMR (Varion T-60 spectrometer) spectra to those prepared from standards.

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