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QUANTITATIVE ANALYSIS OF HYDRALAZINE PYRUVIC ACID HYDRAZONE, THE MAJOR PLASMA METABOLITE OF HYDRALAZINE

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SUMMARY

A specific, high-performance liquid chromatographic technique for the measurement of hydralazine pyruvic acid hydrazone is described. This method utilized reversed-phase chromatography for the separation of this hydrophilic metabolite of hydralazine from other fluid constituents present in serum, plasma, or urine of human volunteers and rabbits receiving hydralazine. Detection of the compound of interest is accomplished spectrophotometrically at 250 nm.

INTRODUCTION

Each of the techniques published over the years concerning the quantitative determination of hydralazine in biological fluids has been non-specific. This applies to the spectrophotometric procedures, employing either ninhydrin[1] or various aromatic aldehydes[2–5] and to the conversion of hydralazine to tetrazolo-phthalazine which may subsequently be determined by gas chromatography (GC)[6]. The non-specificity is due to the fact that hydralazine readily forms hydrazones with endogenous ketones *in vivo* and *in vitro*. Since these hydrazones are unstable at the acidic conditions which have been employed for hydralazine assay, they were partially reconverted to parent drug. As a result the values for hydralazine were found incorrectly high. This fact was first recognized by Zak *et al.*[7], who demonstrated that with decreasing pH, progressively more hydralazine became assayable in plasma of patients treated with hydralazine. Accordingly, they designated the assay results at acidic pH as "apparent" hydralazine. Subsequently, it was shown by Reece *et al.*[8] that a high percentage of "apparent" hydralazine may be composed of the pyruvic acid hydrazone (HPH). This metabolite of hydralazine had been identified earlier in urine[9,10] and plasma[11]. Since the pyruvic acid hydrazone is abundant in both plasma and urine and has been included to an unknown extent on the results obtained from previously non-specific assays, we developed a specific assay which is reported in this paper.

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MATERIALS AND METHODS

Synthesis

The synthesis of HPH has been described by Druey and Ringier[12] and Haegele *et al.*[10]. However, it was not emphasized that the pH of the reaction mixture determines the ratio of two different products which are formed, *i.e.*, the true hydrazone and a tricyclic dehydration product. The true hydrazone forms at pH > 7, and the dehydration product at pH < 7. Formation of the two products is illustrated in Fig. 1. The tricyclic product II is convertible to the hydrazone by addition of base. The hydrazone I cannot be readily converted to the tricyclic product II. However, under acidic conditions, hydrolysis occurs to the starting compounds and additional decomposition products are formed[13].

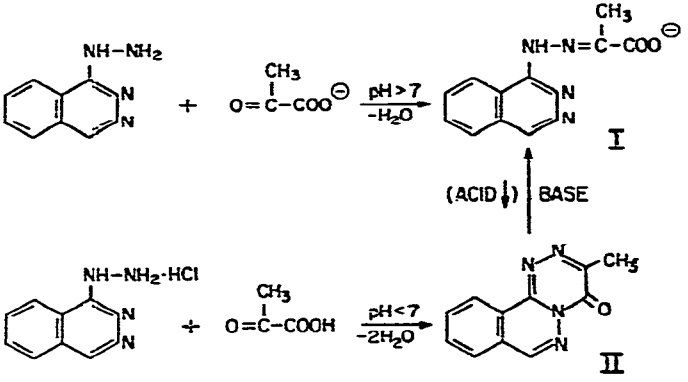

Fig. 1. Formation of true pyruvic acid hydrazone of hydralazine (I) and the tricyclic dehydration product (II).

Synthesis of I. A 5-g amount (0.025 mol) hydralazine hydrochloride (Sigma, St. Louis, Mo., U.S.A.) was dissolved in 50 ml 0.1 *M* sodium phosphate buffer pH 7.4. To this a solution of 11 g (0.1 mol) of sodium pyruvate (Sigma) in 30 ml of the same buffer was added while stirring vigorously. The solution became distinctively yellow almost immediately and the hydrazone precipitated slowly. After standing overnight at 4°, the yellow, crystalline product was filtered off and washed with cold distilled water. The residue was recrystallized from hot ethanol–water. Yield: 4.3 g or 70% of theory.

Synthesis of II. A 5-g amount (0.025 mol) hydralazine hydrochloride was dissolved in 50 ml water and during vigorous stirring 10 g (0.11 mol) of pyruvic acid (Eastman, Rochester, N.Y., U.S.A.) were added, resulting in a reaction mixture of pH 5.5. The light yellow precipitate was collected after a few hours and recrystallized from ethanol–water. Yield: 4.6 g or 85% of theory.

Gas chromatography–mass spectrometry

All newly synthesized materials were routinely analyzed by combined GC–mass spectrometry (MS) (Hewlett-Packard GC–MS computer system 5710A, 5980A and 5933A). Approximately 200 μg of the compounds were dissolved in 50 μl dry pyridine. To this a solution of 50 μl of BSTFA [bis(trimethylsilyl)trifluoacetamide,

Regis, Morton Grove, Ill., U.S.A.] was added and the solution heated in a heating block at 80° for 15 min to effect trimethylsilylation of potentially present starting materials and to form the trimethylsilyl (TMS) ester of I. A 1-μl volume of this solution was then injected onto the GC column (3 ft. × 2.5 mm I.D. coiled glass column, packed with 3% OV-17 on Gas-Chrom Q; Applied Science Labs., State College, Pa., U.S.A.). The column was kept at a starting temperature of 150° and was programmed at 8°/min to 250°. The injector was kept at 250° and the temperature of the ionization source remained at 190–200°. Samples were run in the electron impact (EI) mode of ionization with an ionization energy of 70 eV using helium as the carrier gas (flow-rate 40 ml/min). In the chemical-ionization (CI) mode, methane was used as carrier and reagent gas at a flow-rate of 15 ml/min. For positive proof of the absence and detectability of hydralazine in synthetic I, the sample was spiked on a weight basis with 0.1, 1 and 5% of hydralazine and analyzed analogously. Since 3-methyltriazolo-phthalazine (MTP) was identified as a metabonate arising from I (ref. 13), the amount of MTP present in the samples was quantitated by using d_3-MTP as an internal standard[10].

Assays of HPH by high-performance liquid chromatography (HPLC)

General techniques. Since HPH may potentially exist *in vivo* in forms I and II, it was necessary to develop an analytical procedure allowing determination of both compounds. The instability of I at pH < 7, required that the salt character of I had to be retained throughout any work-up, *i.e.*, the pH had to be > 7. HPLC was chosen to analyze the two compounds. A Varian UV–visible variable-wavelength spectrophotometer with an 8-μl flow cell was used for the detection of these compounds at 350 nm. The detector was interfaced to a Waters Model 6000 dual pump high-performance liquid chromatograph, complete with a Model 6UK septumless loop injector and a Model 3000 solvent programmer for gradient elution. We selected a Waters stainless-steel column (30 cm × 3.9 mm) packed with μBondapak C_{18} (reversed-phase column chromatography). The eluting solvent which resulted in good separation of compounds I and II from serum components was 35% of methanol (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) in 0.1 *M* potassium phosphate buffer pH 7.4 containing 10 m*M* EDTA. The solvent flow-rate was 2.0 ml/min. For regeneration of the column, methanol–water (1:1, v/v) was pumped through the column overnight at a flow-rate of 0.5 ml/min. All solvents were routinely filtered through a 0.45-μm Millipore filter membrane (Millipore, Bedford, Mass., U.S.A.).

Analysis of plasma samples. After hydralazine administration, blood samples from human volunteers and male New Zealand white rabbits were collected in heparinized vacutainers and the plasma separated by centrifugation. To 1 ml of freshly prepared plasma, 1 ml of the phosphate buffer pH 7.4 and 6 ml of methanol were added and thoroughly mixed. This was followed by centrifiugation at 850 *g*. The supernatant was transferred to a 50-ml round-bottom flask and evaporated at 35° on a rotary evaporator. The residue was redissolved in 1 ml eluting solvent and filtered through a 0.45-μm filter in a Sweeney filtration adapter; 100 μl of this reconstituted, filtered solution was then injected onto the liquid chromatographic column via the loop injector. A standard curve in the range 0.1–2.5 μg/ml was obtained analogously by spiking plasma with a freshly prepared solution of I and II in methanol. Potential chromatographic interference of hydralazine and its metabolic products 3-hydroxy-

methyl-triazolo-phthalazine, triazolophthalazine, 4-hydroxy-hydralazine, phthalazine and phthalazinone was investigated, even though none of these products absorbs significantly at 350 nm. Potential chromatographic interference from other hydrazones was examined in spiked buffer eluant, namely the hydrazone derivatives of hydralazine with other abundant endogenous ketones, *e.g.* acetone, acetaldehyde, α-ketoglutaric acid, α-ketoisovaleric acid and α-ketoisocaproic acid.

Analysis of urine samples. Urine samples were processed similarly: 1 ml of urine was mixed with 1 ml of the pH 7.4 buffer solution, followed by 6 ml of methanol. After centrifugation, the supernatant was evaporated to dryness and reconstituted in 1 ml of the eluting solvent. A 100-μl volume was injected onto the chromatographic column. A standard curve was obtained from spiked urine samples in the concentration range of 1 to 500 μg/ml.

Stability of I and II. The chemical stability of I and II was investigated in fresh human and rabbit plasma, as well as in pH 7.4 buffered human urine, rabbit urine (pH 8.0 to 8.5) and in pH 7.4 phosphate buffer alone. The compounds were added separately to yield a final concentration of 1 μg/ml. The peak heights derived from the HPLC analysis were compared as a function of time at 37°.

In vitro formation of I from hydralazine in plasma. The formation of I from hydralazine in spiked human and rabbit plasma was investigated as a function of time. The plasma samples were incubated in a water bath kept at 37° and subsequently analyzed by HPLC.

RESULTS AND DISCUSSION

Purity of synthesized materials is the prerequisite for their use in analytical procedures. In addition these products were used for *in vivo* and *in vitro* testing of hypotensive and vascular smooth muscle relaxant effects, respectively[11]. These applications required close scrutiny for other pharmacologically active products, especially the starting material hydralazine. With the exception of MTP, no impurities were found upon direct-probe MS nor in combined GC–MS using BSTFA as the

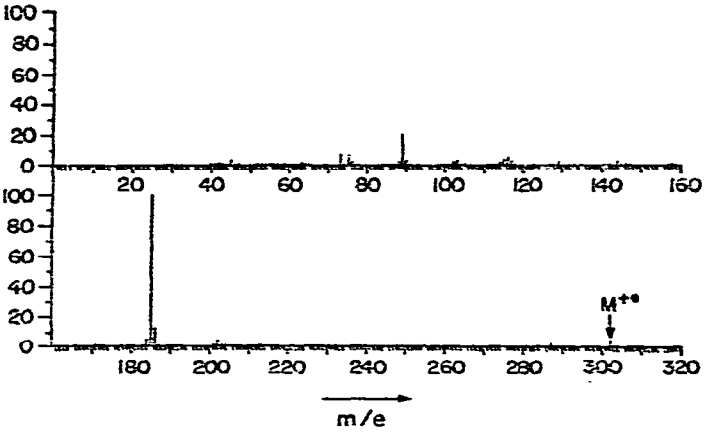

Fig. 2. EI mass spectrum of the pyruvic acid hydrazone of hydralazine (I), TMS ester derivative (Hewlett-Packard 5980 A, 5933 A, GC–MS-computer unit; conditions as described in text).

derivatizing agent for compounds such as hydralazine. MTP was present in I at a level of 0.78% (w/w). Spiking I and II with 0.1, 1 and 5% of hydralazine yielded clearly detectably levels of hydralazine. Based on this calibration, compound I contained 0.03% (w/w) hydralazine and none was detectable in compound II. Fig. 2 shows the EI spectrum of I, with *m/e* 302 as the molecular ion of the TMS ester derivative. Loss of ·COOTMS results in the base peak of the spectrum at *m/e* 185. Fig. 3 shows the EI spectrum of II, with the molecular ion and base peak at *m/e* 212. This molecular weight is confirmed by CI, producing $MH^{\oplus}$ at *m/e* 213, $MC_2H_5^{\oplus}$ at *m/e* 241 and $MC_3H_5^{\oplus}$ at *m/e* 253. Elimination of CO from *m/e* 212 yields *m/e* 184. The genesis of other fragment ions has been discussed previously[10].

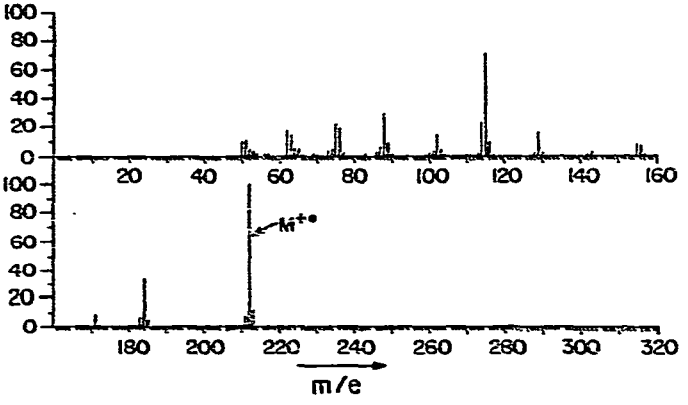

Fig. 3. EI mass spectrum of the tricyclic dehydration product formed from hydralazine and pyruvic acid at pH 4.5 (II).

The stability of I and II was examined in phosphate buffer, plasma and urine by spiking the various fluids with 1 μg/ml. The urine was diluted 1:1 with pH 7.4 buffer to adjust the pH. Samples were analyzed at 5, 15, 30, 60, 90, 120 and 240 min and did not show any decomposition of I or II as judged by the corresponding peak heights resulting from the HPLC analysis. During incubation, samples were shielded from light by using aluminum foil. Without this protection significant and irreproducible loss of I was observed. Stability studies of I in acidic buffers, pH 6.2 and 5.4, resulted in considerable loss of I (Fig. 4) as measured over a time period of 4 h. It was further determined that no chromatographic interference occurred from other metabolites or proposed metabolites of hydralazine (see *Analysis of plasma samples*).

Fig. 5a shows a calibration curve, obtained by injection of increasing amounts of a solution of I in HPLC eluting solvent. The standard curve showed excellent linearity over the range of 10 ng to 10 μg (the range of 10 ng to 1 μg is pictured only, due to space limitation). Fig. 5b shows the calibration curve obtained from spiked human plasma over a range of 100 ng/ml to 2.5 μg/ml. Minimum detectable concentration ranged from 10 to 25 ng/ml of I in human or rabbit plasma and from 50–100 ng/ml in urine, with a peak-to-noise ratio of 3:1. Table I illustrates the mean and dispersion around the mean of I as determined for spiked plasma samples on six consecutive days.

A typical chromatogram, showing the analysis of I and II in a human plasma

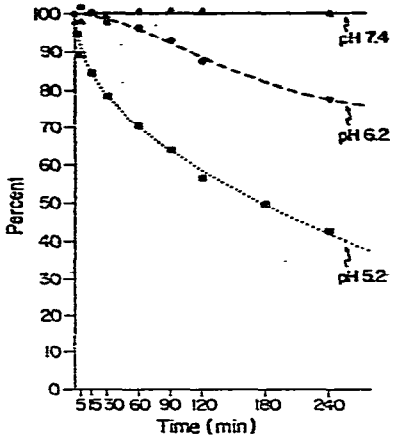

Fig. 4. Stability of I in different buffer systems as a function of time.

sample spiked with 200 ng/ml of each is shown in Fig. 6a. A blank plasma is shown in Fig. 6b. Fig. 6c shows the HPLC chromatogram obtained from a plasma sample of a human volunteer, who had received 1 mg/kg bodyweight of hydralazine hydrochloride orally. The sample was collected 35 min after dosing. The concentration of I was determined to be 630 ng/nl. It is evident that no peak corresponding to compound II was observed in Fig. 6c. In addition, no peak for II was observed in any other urine or plasma sample after either intravenous or oral administration of hydralazine to six human volunteers. Accordingly compound I, but not compound II, is a metabolite of hydralazine in humans. Absence of II was also established in plasma and urine of rabbits when dosed with hydralazine intravenously.

The ease of formation of I in plasma is illustrated in Fig. 7. Rabbit plasma was incubated at 37° with hydralazine hydrochloride at a concentration of 10 μg/ml. Aliquots for analysis were taken at 5, 15, 30, 60 and 120 min and processed for HPLC analysis. The rapid formation of I is evident since even at 5 min, I is already present in the sample. At 120 min a 33.5% conversion of hydralazine to I had oc-

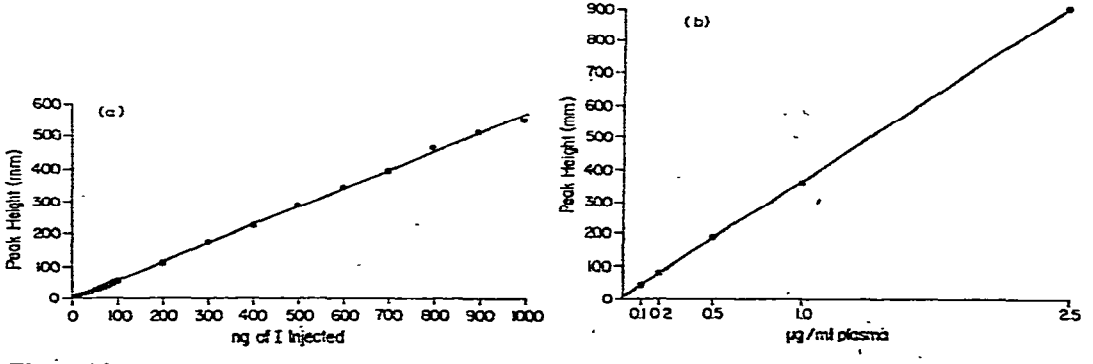

Fig. 5. (a) HPLC calibration curve obtained by injection of increasing amounts of I dissolved in the eluting buffer system. (b) HPLC calibration curve of I obtained form spiked human plasma samples.

TABLE I

PEAK HEIGHT (mm)

Peak height is normalized for an absorbance range of the UV–VIS recorder of 0.05.

Amount of I added to 1 ml plasma (μg)	*Mean* $\bar{X}$	*Standard deviation (S.D.)*	*Number of analyses (n)*	*Coefficient of variation (%)* $\frac{S.D.}{\bar{X}} \cdot 100$
0.1	4.37	0.2888	6	0.6
0.2	7 86	0.114	5	1.5
1.0	36.25	1.317	6	3.6
2.5	90.67	2.724	6	3.0

curred calculated on the basis of mol-to-mol conversion. It was documented that the addition of methanol and subsequent workup of the samples did not prevent the further formation of I from the hydralazine present. The incubation of hydralazine for 120 min with two samples of human plasma obtained from different donors yielded 79.5 and 50.8% molar conversion of hydralazine to I, when hydralazine was present in initial concentrations of 2.5 and 10 μ/ml, respectively. Different yields of I obtained after incubation of the plasma samples may reflect differences in the excess of pyruvic acid present, in respect to the concentration of added hydralazine.

In practice, the continuing formation of I from hydralazine was shown not to pose a significant problem for the determination of I in the plasma of human volunteers receiving orally administered hydralazine hydrochloride. Continued formation of I from hydralazine during workup would not make a major contribution to I, since

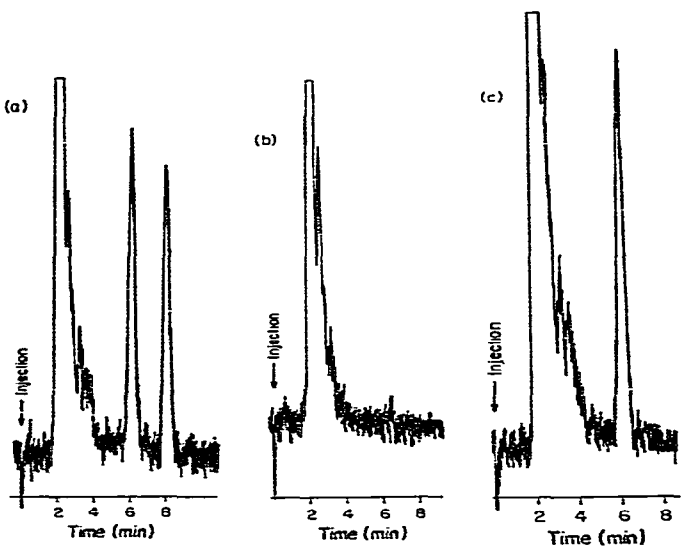

Fig. 6. (a) HPLC chromatogram obtained from a human plasma spiked with 200 ng/ml each of I and II. Injection corresponds to 1/10 of the total, reconstituted solution obtained after workup. (b) HPLC chromatogram obtained from a blank human plasma sample. (c) HPLC chromatogram of a human plasma sample collected from a volunteer 35 min after oral administration of 1 mg/kg bodyweight of hydralazine hydrochloride.

(a) hydralazine was present in concentrations which were considerably lower than previously reported[14], (b) hydralazine became undetectable in plasma 45 min after dosing[15], while I was present in measurable concentrations for at least 6 h (please refer to Fig. 8) and (c) the hydralazine level in human volunteers never exceeded 15% of the concentration of I when measured 20 min after dosing[14,15].

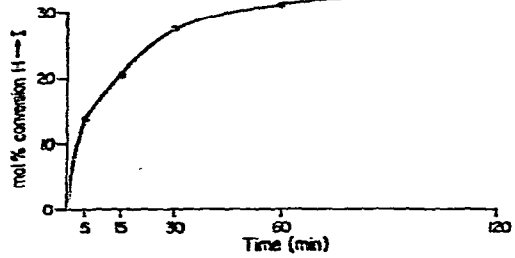

Fig. 7. *In vitro* formation of I in rabbit plasma, after spiking the plasma with 10 μg/ml of hydralazine hydrochloride.

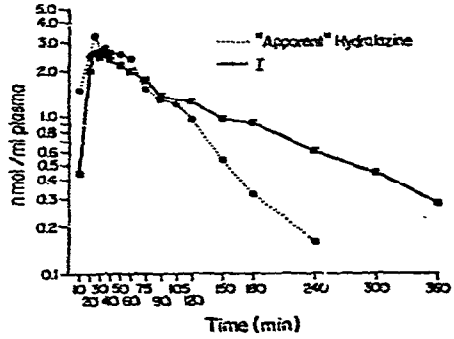

Fig. 8. Time course of I in plasma after oral administration of 1 mg/kg bodyweight hydralazine hydrochloride to a male, human volunteer.

Fig. 8 illustrates the time course of I in plasma of a human volunteer. Serial plasma samples were collected for 6 h after hydralazine intake and were analyzed as described. Levels of I reached a peak concentration of 2.5 nmol/ml or 655 ng/ml at 25 min after dosing. The concentration of I subsequently rapidly declined in a first order fashion with a half life of 106 min. "Apparent" hydralazine plasma levels were also determined using methods described[6,8]. It is evident that the relative ratios of "apparent" hydralazine to I were not constant but changed over a wide range. The decay of "apparent" hydralazine was first order, with a half-life of 47 min. From these findings, it is evident that the decay of "apparent" hydralazine does not parallel the decay of I and therefore is no a good measure of the concentration of I in body fluids, again illustrating the requirement for specific methods.

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