IDENTIFICATION OF HYDRALLAZINE AND HYDRALLAZINE HYDRAZONE METABOLITES IN HUMAN BODY FLUIDS AND QUANTITATIVE *IN VITRO* COMPARISONS OF THEIR SMOOTH MUSCLE RELAXANT ACTIVITY

K.D. HAEGELE^{1,2}, A.J. McLEAN^{1,2}, * P. du SOUICH^{1,2}, K. BARRON², J. LAQUER³, J.L. McNAY^{1,2} & O. CARRIER²

¹ Division of Clinical Pharmacology and Hypertension,

² Departments of Medicine and Pharmacology and

³ Division of Ambulatory Care, Department of Family Practice;

Audie Murphy Veterans Administration Hospital and

University of Texas Health Science Center, San Antonio, Texas 78284, USA

1 Serum and urine from hypertensive subjects on chronic oral hydrallazine therapy were studied using gas chromatographic/mass spectrometry techniques.

2 Metabolites resulting from acetylation, hydrolysis and conjugation reactions were detected. The acetone, pyruvate and α -ketoglutarate hydrazone were identified.

3 The activity of the pyruvate and α -ketoglutarate hydrazones were compared with that of hydrallazine using isolated rabbit aortic strips.

4 Both hydrazones were active under *in vitro* conditions, producing smooth muscle relaxant effects equal to those of hydrallazine.

5 It is concluded that hydrazone metabolites will contribute to the hypotensive effects of hydrallazine therapy in proportion to their relative abundance, persistence in vascular tissues and intrinsic activity.

Introduction

Studies on urine from animals and man have revealed a variety of products derived from hydrallazine (H). The results of these studies have been summarized in comprehensive metabolic schema published previously (Haegele, Skrdlant, Robie, Lalka & McNay, 1976; McLean, Haegele, du Souich & McNay, 1977). Because of the instability of H (Schulert, 1961; Talseth, 1976), the possibility has not been rigorously excluded that significant proportions of these products may represent metabonates formed in the urinary tract.

There do not appear to have been any previous reports of the identification of H metabolites in plasma, however a recent study provides evidence for the presence of acid-labile metabolites in human and rat plasma (Zak, Lukas & Gilleran, 1977) without identifying these substances. Because these products revert to H under acid conditions, they almost certainly represented hydrazones (Dayton & Israili, 1977). The only metabolically derived hydrazones described to date are the pyruvate (Haegele *et al.*, 1976; McIsaac & Kanda, 1964) and acetone

hydrazones (Haegele *et al.*, 1976), however a number of other potential products have been proposed (Dayton & Israili, 1977).

The purpose of this study was to apply gas chromatography/mass spectrometry techniques (Haegele *et al.*, 1976; McLean *et al.*, 1977) to the identification of H or its products in human plasma and urine and to assess the biological activity of any potentially active products detected.

Methods

Specimen collection and analysis

Blood and urine samples were obtained from hypertensive subjects on chronic therapy with H, 1–5 h after the morning dose. The subjects studied were free of significant cardiac, renal or hepatic disease and were receiving daily dosages ranging from 30–400 mg/day.

3-Pentanone was added to serum (0.2 ml/ml) and urine (0.3 ml/100 ml) samples as soon as practicable (<20 min) after collection. Samples were then divided into two parts. One part was handled as described

* Present address: Department of Pharmaceuticals, School of Pharmacy, C531 Cooke-Hochstetter, Amherst, New York 14260, USA

Figure 1 Mass spectra of hydrallazine pentanone hydrazone.

previously (Haegele *et al.*, 1976). The other part was extracted into CHCl_3 /isopropanol without acidification, then processed as before.

Synthesis and validation of test metabolites

The synthesis of the pyruvate hydrazones (HPH) has been published previously (Haegele *et al.*, 1976).

Hydrallazine α -ketoglutarate hydrazone (HKH). Hydrallazine hydrochloride (395 mg; 2 mmol) was dissolved in 5 ml of water. To this an aqueous solution of α -ketoglutaric acid (1 g; 7 mmol) was added and the reaction mixture was allowed to stand overnight at room temperature. The solid precipitate was filtered off and dried *in vacuo*. Yield 510 mg or 88% theory.

Synthetic products were validated by combined gas chromatography/mass spectrometry on a Hewlett-Packard system (Haegele *et al.*, 1976; McLean *et al.*, 1977).

In vitro pharmacological techniques

The relative potency of each of the hydrazones was tested using an isolated rabbit aortic strip technique described previously (Carrier, Wedell & Barron, 1977; Barron, Carrier, Haegele, McLean, McNay & du Souich, 1977). Four equivalent strips from each aorta were available from any one animal and paired comparisons were made between each of the hydrazones and hydrallazine.

Strips were contracted by replacement of Locke solution (Carrier *et al.*, 1977, Barron *et al.*, 1977) by a solution with 30 mM K^+ ($\text{Na}^+ \rightarrow \text{K}^+$ substitution in the Locke medium). Test compounds were added cumulatively to the bath over the test range 10^{-6} – 5×10^{-4} M. The relaxant effects were measured at each dose and expressed as a percentage of the tension generated in the K^+ contracture. Comparisons were made using paired *t*-testing.

Stock solutions of each test hydrazone were made up with ethanol and DMSO. Test and control strips were each exposed to the same concentrations of drug vehicle.

Results

Detection of hydrallazine metabolites in human serum and urine

H was detected as the pentanone derivative (Figure 1), along with a range of other products (Table 1). The amounts and proportions of each product varied over a wide range, possibly reflecting the wide variation in dose schedules, variation in dose-sampling intervals, sex and organ function of the subjects studied.

Precise quantitation will require standardization of subjects, sample collections and dosage schedules and will be the subject of a future report.

Figure 2 Mass spectra of hydrazaline α -ketoglutaric acid hydrazone. a Chemical ionization, methane; b Electron impact.

Table 1 Metabolic products of hydrazaline identified in human body fluids

	Urine	Serum
Parent drug	+	+
Phthalazine	+	+
Phthalazinone	+	+
*MTP	+	+
TP-CH ₂ OH	+	—
TP-COOH	+	—
**TP	+	—
4-OH-MTP	+	—
Hydrazones:		
Acetone (HAH)	+	+
Pyruvic acid (HPH)	+	+
α -Ketoglutaric acid (HKH)	+	+

*3-Methyl-s-triazolo[3,4 α]phthalazine
 **s-triazolo[3,4 α]phthalazine

Mass spectra of test hydrazones

HPH and HKH standards were validated by GC-MS. Spectra of HPH have been published previously (Haegele *et al.*, 1976; McLean *et al.*, 1977). HKH showed a GC retention time of 6.7 min and the mass spectra shown in Figure 2.

In order to establish the molecular weight of this compound, chemical ionization mass spectrometry was chosen with methane as the reactant gas (Figure 2a). The series of adduct ions ($MH^+ = 271$; $MC_2H_5^+ = 299$; $MC_3H_5^+ = 311$) confirms the molecular weight of this compound to be 270. Elimination of H_2O from MH^+ yields the ion at m/e 253. Upon electron impact mass spectrometry (ionization energy 70 eV), spectrum in Figure 2b was obtained. The molecular ion M^+ is seen at m/e 270. Loss of $\cdot OH$ and H_2O from the molecule yields the

Figure 3 Illustration of the relaxant effects of hydrallazine (H) and hydrazone derivatives on K^+ -induced contractions of rabbit isolated aortic strips.

ions at m/e 263 and 262 respectively. Expulsion of $\cdot\text{COOH}$ leads to the base peak at m/e 225, loss of $\cdot\text{CH}_2\text{COOH}$ and $\cdot\text{CH}_2\text{CH}_2\text{COOH}$ gives rise to the ions at m/e 211 and 197, respectively. The ion due to the phthalaziny moiety is observed at m/e 129. Elimination of HCN from this ion then forms m/e 102. Thus mass spectrometry confirms the structure of this compound to be the cyclic dehydration product of the α -ketoglutaric acid hydrazone of hydrallazine.

This is in accordance with the dehydration and cyclization described for the pyruvic acid hydrazone by Druey & Marxer (1959), and illustrated by its mass spectra by Haegele *et al.* (1976). This cyclization is reversed at alkaline pH and the regular hydrazone is observed.

In vitro comparisons of the relative potency of H, HPH and HKH

HPH and HKH each caused dose-dependent relaxation of K^+ -induced contractures (Figure 3). The relaxant effects were similar to those of parent H. Quantitative comparisons derived from studies on paired strips of five aortae are shown in Figure 4. There were no significant differences between H and HKH over the test range, however, the activity of HPH exceeded that of HKH at the 5×10^{-4} M dose level ($P < 0.05$).

Discussion

The detection of a spectrum of metabolites of H in human body fluids is important. Most significantly, the serum findings establish that these metabolites are not artifacts of incubation in the urinary tract. This possibility has been considered particularly applicable to hydrazone derivatives (Druey & Tripod, 1967). These results also indicate that the same kinds of metabolic products are formed in man as described previously for experimental animals.

This report lacks quantitation of the relative or absolute amounts of H and hydrazone products found, however this information is contained in a recent work by Zak and co-workers (1977). In this study humans were dosed with 50 mg H orally. It was found that acid-labile products (i.e. hydrazones) were present in amounts equal to 2–3 times that of parent H at peak, and of the order of five times the amounts of H at 4 h. In this setting, the findings in this paper assume significance.

The analytical results suggest that the components which make up this hydrazone pool are limited to the acetone, pyruvate and α -ketoglutarate conjugates, as detection threshold for the GC–MS technique makes it unlikely that other products of this kind are present unless they do not extract into organic solvents from plasma samples.

Figure 4 Quantitative comparisons of the relaxant effects of hydrazine and hydrazone derivatives on rabbit aortic smooth muscle. Each point represents the mean of results in six aortae, the bars represent s.e. mean. Δ Hydrazine (H); $\circ$ Hydrazine pyruvate hydrazone (HPH); $\bullet$ Hydrazine α -ketoglutarate hydrazone (HKH). * $P < 0.05$.

There have been few previous reports of quantitative testing of the activity of H metabolites. Past testing of compounds related to H was qualitative and involved systemic administration to anesthetized rabbits (Druey & Marxer, 1959; Druey & Tripod, 1967). Hydrazone derivatives were tested and found to have activity equivalent to that of parent H (Druey & Tripod, 1967). No other groups of derivatives had

activity approaching that of H. Recent studies have established that HAH is active under *in vivo* (McLean *et al.*, 1977) and *in vitro* (Barron *et al.*, 1977) conditions. The activity of HAH was reported to exceed that of H (Barron *et al.*, 1977), and it was shown that H was stable (i.e., no phthalazine or phthalazinone was detected). The data presented here on the relative potency of H, HPH and HKH indicate that these hydrazones are at least as active as parent H.

Because hydrazones are quantitatively more significant than H (even at peak), and because these compounds are more persistent (suggesting a longer $T_{1/2}$ than H), it is likely that hydrazones will accumulate under chronic dosing regimens. The analytical results presented suggest that only HAH, HPH and HKH are likely to be present. Consequently results relating to relative potency of HPH and HKH compared to H are important, as it allows the initial conclusion that these products along with HAH have the potential to contribute to the hypotensive effects which follow H administration.

These results point to the need both for quantitative studies of metabolism and parallel quantitative testing of metabolite efficacy to further assess the possibility that active metabolites of H may significantly contribute to the hypotensive effects of H in animals and man. Further interest in the differential pharmacological activity of metabolite stems from the possibility that hypotensive effects may be separable from any capacity to interact with the immunological processes mediating hydrazine-related lupoid syndrome (Perry, 1973).

Supported in part by a Fellowship from the National Health and Medical Research Council of Australia (AJMcL), by a Merck, Sharp and Dohme International Fellowship in Clinical Pharmacology (P.d.S.) and by the Veterans Administration. Reprint requests should be addressed to AJMcL.

References

- BARRON, K.W., CARRIER, O., HAEGELE, K.D., McLEAN, A.J., McNAY, J.L. & DU SOUICH, P. (1977). Comparative evaluation of the *in vitro* effects of hydrazine and hydrazine acetone on arterial smooth muscle. *Br. J. Pharmac.*, **61**, 345–349.
- CARRIER, O., WEDELL, E.K. & BARRON, K.W. (1977). Specific α -adrenergic receptor desensitization in vascular smooth muscle. *Blood Vessels* (in press).
- DAYTON, P.G. & ISRAELI, Z.H. (1977). Metabolism of hydrazine. *Drug Metab. Rev.* (in press).
- DRUEY, J. & MARXER, A. (1959). Hypotensive hydrazinophthalazines and related compounds. *J. Med. Pharm. Chem.*, **1**, 1–21.
- DRUEY, J. & TRIPOD, J. (1967). *Medicinal Chemistry* **7**, 223–262. New York and London: Academic Press.
- HAEGELE, K.D., SKRDLANT, H.B., ROBIE, N.W., LALKA, D. & McKAY, J.L. (1976). Determination of hydrazine and its metabolites by gas chromatography-mass spectrometry. *J. Chromatogr.*, **126**, 517–534.
- McISAAC, W.M. & KANDA, M. (1964). The metabolism of 1-hydrazinophthalazine. *J. Pharmac. exp. Ther.*, **143**–144, 7–13.
- McLEAN, A.J., HAEGELE, K.D., DU SOUICH, P. & McKAY, J.L. (1977). Comparative evaluation of the hypotensive activity of two major metabolites of hydrazine (1-hydrazinophthalazine). *Eur. J. Drug Metab. Pharmacokin.*, **1**, 17–20.
- PERRY, H.M., JR. (1973). Late toxicity to hydrazine resembling systemic lupus erythematosus or rheumatoid arthritis. *Am. J. Med.*, **54**, 58–72.

- SCHULERT, A.R. (1961). Physiological disposition of hydralazine and a method for its determination in biological fluids. *Arch. Int. Pharmacodyn.*, **132**, 1–15.
- TALSETH, T. (1976). Studies on hydralazine. II. Elimination rate and steady state concentration in patients with impaired renal function. *Eur. J. clin. Pharmac.*, **10**, 311–317.
- ZAK, S.B., LUKAS, G. & GILLERAN, T.G. (1977). Plasma levels of real and 'apparent' hydralazine in man and rat. *Drug Metab. Disp.*, **5**, 116–121.

(Received June 15, 1977)