FURTHER EVIDENCE FOR AN ACETYLATOR PHENOTYPE DIFFERENCE IN THE METABOLISM OF HYDRALAZINE IN MAN

VINCENZO FACCHINI & JOHN A. TIMBRELL

Clinical Toxicology Unit,
Department of Clinical Pharmacology,
Royal Postgraduate Medical School,
Ducane Road, London W12 0HS

- 1 The 0-24 h urine from hypertensive patients treated with hydralazine (100 mg twice daily) has been analysed by gas chromatography and high pressure liquid chromatography.
- 2 4-*N*-Acetylhydrazinophthalazine-1-one (NAcHPZ), *s*-triazolo [3,4-*a*] phthalazine (TP), phthalazinone (PZ) and hydralazine (free, H; acid-labile hydrazones, HH) were detected and assayed.
- 3 The results indicate that slow acetylators excrete less NAcHPZ and TP than rapid acetylators but more PZ and HH.
- 4 Free hydralazine was present in low levels and was only detected in some urine samples.
- 5 The ratios of the metabolites NAcHPZ/HH; TP/HH; NAcHPZ/PZ and PZ/TP are different in the two acetylator phenotypes.
- 6 It is possible the ratio PZ/TP may be used for determination of acetylator phenotype.
- 7 It is concluded that hydralazine metabolism is dependent on the acetylator phenotype.

Hydralazine (1-hydrazinophthalazine, H) is a widely used antihypertensive drug (Koch-Weser, 1979). Soon after its introduction the drug was reported to cause a syndrome that closely resembled systemic lupus erythematosus (Perry & Schroeder, 1954). This condition can occur in up to 12% of recipients on high doses (Perry, 1973). The manifestations are usually reversible following withdrawal of the drug, and antihydralazine antibodies and antinuclear antibodies are demonstrable (Perry, 1973). Predisposing factors in the development of the condition are prolonged therapy, high dosage regimes and the slow acetylator phenotype (Perry, Tan, Carmody & Sakamoto, 1970). Consequently it has been suggested that differences in metabolism between rapid and slow acetylators particularly at high doses may play an important role in producing the condition.

The metabolism of hydralazine in man is complex (Figure 1) and known to involve at least two acetylation pathways (McIssac & Kanda, 1964; Wagner, Faigle, Imhof & Leil, 1977; Zimmer, McManus, Novinson, Hess & Litwin, 1970). The formation of methyltriazolol [3,4-*a*]phthalazine (MTP) (Talseth, 1976) and 3-hydroxy methyltriazolo [3,4-*a*]phthalazine (OH.MTP), products of *N*-acetylation followed by ring closure is under the same genetic control as the acetylation of sulphamethazine, and the ratio OH.MTP/hydralazine hydrazones

(HH) shows a bimodal distribution similar to that for the ratio of acetylsulphamethazine/sulphamethazine (Timbrell, Harland & Facchini, 1980).

As the lupus erythematosus-like syndrome is almost exclusively confined to slow acetylators and as hydralazine is itself polymorphically acetylated in man (Timbrell *et al.*, 1980) it is conceivable that the condition may arise through differences in the metabolism of the compound by slow and rapid acetylators. Saturation of the acetylation pathway in slow acetylators particularly after high doses could result in the channeling of the drug through alternative pathways. This could result in higher levels of non-acetylated metabolites. Such metabolites might be involved in the development of the lupus syndrome.

It was the aim of this study initially to determine the levels of known metabolites of hydralazine in the 24 h urine of patients receiving a twice daily 100 mg dose in order to ascertain whether significant differences in the levels of these metabolites existed between rapid and slow acetylators. Consequently the terminal product of the primary acetylation pathway; *s*-triazolo [3,4-*a*]phthalazine (TP), the products of alternative pathways; 4-hydrazinophthalazin-1-one (HPZ), 4-*N*-acetylhydrazinophthalazin-1-one (NAcHPZ) and phthalazinone (PZ) were measured to further define the role of phenotype

Figure 1 Metabolism of hydralazine in man.

status in hydralazine metabolism. The determination of certain metabolic ratios notably NACHPZ/HH, TP/PZ and NACHPZ/PZ might also be used for acetylator phenotyping patients without recourse to sulphamethazine which involves the inconvenience of temporarily stopping hydralazine therapy.

Methods

Clinical procedure

The patients studied were attending the Hypertension Clinic at Hammersmith Hospital and were undergoing maintenance therapy with hydralazine, receiving a twice daily dose of 100 mg at approximately 08.00 h–10.00 h and 20.00 h–22.00 h. Urine was collected for 24 h after the initial dose in bottles containing 10 ml of 2M HCl; added in order to minimise the breakdown of hydralazine reported to occur at more alkaline pH values (Schulert, 1961). The average final pH was 5.3 ± 1.47 (mean $\pm$ s.d.)

The patients had been receiving in conjunction with hydralazine a number of other drugs: (numbers of patients indicated): clorethiazole 1⁺; bendroflumazide 8; atenolol 7; clonidine 1⁺; glyceryl trinitrate 1⁺; propranolol 6; methyl dopa 6; oxprenolol 2; digoxin 2⁺; frusemide 2; naproxen 1⁺; diazepam 1⁺; navidrex 2⁺; moduretic 4; Slow K 7; bethanidine 2 and hydrochlorothiazide 1⁺ (*These drugs were only administered to rapid acetylators; *these drugs were only administered to slow acetylators). Patients' acetylator status was determined by the sulphamethazine method (second dose schedule) (Evans, 1969). Creatinine levels were determined by the alkaline picrate method, mean $\pm$ s.d. g 24 h⁻¹: 1.47 ± 0.47 (males); 1.07 ± 0.38 (females).

Materials

Hydralazine was obtained from Koch-Light Laboratories, and acetylacetone from BDH Chemicals.

HPZ, NACHPZ, TP, PZ and methylhydralazine

(MeH) were generously supplied by Ciba-Geigy Ltd, Basel, Switzerland.

β -Glucuronidase containing sulphatase activity (type H1, from *Helix pomatia*) was obtained from Sigma Chemical Co.

Gas chromatography (GC)

Determinations by GC were carried out on a Perkin Elmer F17 instrument, fitted with a nitrogen-phosphorous detector. The glass column (2 m $\times$ 1.75 mm i.d.) was packed with 10% OV-17 on Gas Chrom Q, 100–120 mesh (Applied Science). The oven and detector/injector temperatures were set at 250 and 300°C respectively, with a nitrogen carrier gas flow rate of 35 ml min⁻¹.

High pressure liquid chromatography (HPLC)

HPLC determinations were performed following the injection of 5 μ l aliquots of urine extracts (Waters U6K injector) onto a reverse phase ODS-hypersil column, 25 cm $\times$ 5 mm i.d. (Shandon Southern Products, UK). Solvent delivery of methanol/water, 15/85 (v/v) at a flow rate of 1.8 ml min⁻¹ (TP, PZ assay), and methanol/water, 50/50 (v/v) at a flow rate of 1.3 ml min⁻¹ (free H assay) was effected by a Waters 6000A pump. Monitoring of the eluent was performed on a Pye Unicam variable wavelength LC-UV detector, set at 254 nm.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Finnigan 3200 instrument. The GC was fitted with a 5ft $\times$ 2 mm i.d. glass column packed with 10% OV-17 on Gas Chrom Q, 100–120 mesh. Helium at a flow rate of 30 ml min⁻¹ was used as the carrier gas, with an oven temperature of 240°C (PZ, TP, NAcHPZ, HPZ analysis) and 260°C (analysis of methylated 4-hydrazinophthalazin-1-one pyruvate hydrazone). The electron energy of the GC-MS was set at 25 eV.

Syntheses

4-Methylphthalazin-1-one (MePZ) was synthesised as previously described (Facchini & Timbrell, 1980).

4-Hydrazinophthalazin-1-one pyruvate hydrazone a possible metabolite of hydralazine was synthesised by reacting equimolar amounts of 4-hydrazinophthalazin-1-one and sodium pyruvate at 30°C, pH 6 in H₂O for 2 h. The pale yellow precipitate which resulted was filtered, dissolved in H₂O at pH 8.0 and extracted twice with methylene chloride. The hydrazone was reprecipitated by adjusting to pH 6.0, filtered and then dried in a dessicator. The hydrazone proved to be unsuitable for GC and was derivatised by reaction with diazomethane for 5 days at room

temperature to give a single methylated derivative on GC. GC-MS analysis of the derivative gave a single peak, retention time 2.85 min and a molecular ion (M⁺) of m/e 288, and a fragmentation pattern; m/e 245 (M-43), m/e 229 (M-59, base peak), m/e 188 (M-100), m/e 159 (M-129), m/e 156 (M-132), m/e 130 (M-158), m/e 129 and (M-159) consistent with the structure of a trimethyl derivative of 4-hydrazinophthalazin-1-one pyruvate hydrazone.

Assay procedures

(*Acid labile hydralazine*) (HH) HH was measured by the method previously described (Facchini, Streeter & Timbrell, 1980). Duplicate urine samples, pre-extracted at pH 9.5 to remove MTP which interferes with the assay, were adjusted to pH 0.1. Under these acidic condition the hydrazones release free hydralazine, which can then be measured as the pyrazole derivative after reaction with acetylacetone (Smith, Johnson & Kho, 1977). The internal standard curve was prepared by spiking blank urine with H and MeH, reacting with acetylacetone at pH 0.1 and then extracting into methylene chloride for subsequent GC assay. This was carried out at the same time as the patients' urines determinations.

(*Free hydralazine*) (H) Duplicate urine samples (10 ml) were adjusted to pH 7.4 (hydralazine hydrazones are not hydrolysed at this pH) and acetylacetone (0.5 ml) and the internal standard MeH added. After reaction for 1 h at room temperature the urine was adjusted to pH 9.5 and extracted with methylene chloride (30 ml). The extract filtered through phase separating paper was reduced to dryness and dissolved in methanol (1.0 ml). Aliquots of this methanolic solution were analysed by HPLC under conditions described above.

A standard curve was prepared by spiking blank urine with hydralazine and methylhydralazine and assaying as described above.

(*4-Hydrazinophthalazin-1-one*) (HPZ) HPZ was determined by GC in duplicate extracts of patients' urines, after derivatisation with acetylacetone at pH 3.0 to form a pyrazole derivative as previously described (Facchini & Timbrell, 1980). GC conditions are given above. The internal standard used was MeH. A standard curve prepared by spiking blank urine with HPZ and MeH was assayed at the same time and under identical conditions to patients' urine samples. The synthesised pyruvate hydrazone of HPZ (a possible metabolite of H in man) was shown to be 100% hydrolysed at pH 3.0 under the conditions of this assay. The assay will therefore measure free HPZ and its hydrazones. As previously described NAcHPZ was not hydrolysed at pH 3.0 and therefore did not interfere in the assay.

4-*N*-Acetylhydrazinophthalazine-1-one (NAcHPZ)
This metabolite was determined after acid hydrolysis to yield HPZ which was reacted immediately with acetylacetone to produce a pyrazole derivative as described above. MeH was used as the internal standard.

Duplicate urine samples and a calibration curve (prepared by spiking blank urine with NAcHPZ and MeH) were processed under identical conditions at the same time, as previously described (Facchini & Timbrell, 1980), and determined by GC under the conditions given above. Any HPZ present before hydrolysis will also be measured but it can be determined separately as described above and the value subtracted from the total.

Phthalazine and *s*-Triazolo [3,4-*a*]phthalazine (PZ and TP) These metabolites were determined simultaneously underivatized, by HPLC using MePZ as the internal standard (Facchini & Timbrell, 1980).

PZ and TP were determined in extracts of duplicate urine samples by HPLC as previously described. The chromatographic conditions used are stated above. A standard curve was prepared by spiking blank urine with TP, PZ and MePZ. This was carried out at the same time as the assay of patients' urines. Urines were also assayed for PZ after treatment with β -glucuronidase/sulphatase. This involved the incubation of urines with β -glucuronidase/sulphatase for 16 h at pH 5.0 in 0.1M acetate buffer. Control incubations contained no enzyme.

Results

NAcHPZ assayed after acid hydrolysis, as the pyrazole derivative of HPZ was detected in the 0-24 h urines of patients treated with hydralazine. No HPZ was detected in non-hydrolysed urines. GC-MS analysis confirmed that the peak, retention time 11.6 min which was not present in blank urine, was 4-(3,5-dimethylpyrazole)phthalazin-1-one. The compound had a molecular ion and fragmentation pattern

identical with the authentic (standard extracted from urine); m/e (M^+) 239 (molecular ion and base peak; ($M^+ + 1$) m/e 240; ($M^+ - 41$) m/e 198; ($M^+ - 96$) m/e 143; ($M^+ - 109$) m/e 130; ($M^+ - 143$) m/e 96. It was concluded that NAcHPZ was present in urines investigated.

Analysis of urine extracts by HPLC revealed the presence of two peaks with retention times identical to those for PZ and TP of 14 and 23.5 min respectively. These peaks were not present in blank urine.

The peak corresponding to PZ in the urine extracts was collected and purified by preparative chromatography on an ODS-hypersil column under the conditions described above. GC-MS analysis gave a single peak, retention time 1.60 min with a molecular ion (M^+) m/e 146 (base peak) and a fragmentation pattern of ($M^+ + 1$) m/e 147, ($M^+ - 27$) m/e 119, ($M^+ - 55$) m/e 91, ($M^+ - 56$) m/e 90 and ($M^+ - 57$) m/e 89 identical to that of authentic PZ (retention time 1.64 min). It was concluded that PZ was present in the urines investigated.

Urine extracts containing the peak corresponding to TP were analysed directly by GC-MS under the conditions described above. The retention time of 6.32 min for this peak exactly corresponded to the retention time of authentic TP. Mass spectral analysis of this peak gave a molecular ion m/e 170 (base peak) and fragmentation pattern ($M^+ + 1$) m/e 171, ($M^+ - 54$) m/e 116, ($M^+ - 55$) m/e 115, ($M^+ - 57$) m/e 114 and ($M^+ - 82$) m/e 88, which was identical to that of authentic TP. It was concluded that TP was present in the urines investigated.

Metabolite levels

The levels of NAcHPZ, TP, HH, H and PZ in the 0-24 h urine of patients given a twice daily dose of 100 mg hydralazine are shown in Table 1.

The level of the acetylated metabolites NAcHPZ and TP are higher in the rapid compared with the slow acetylator phenotype although the phenotype

Table 1 Excretion of metabolites in the 0-24 h urine of patients given 100 mg of hydralazine twice daily

Acetylator phenotype	H	HH (1)	PZ (2)	TP (3)	NAcHPZ (4)
Rapid (7)	0.87 $\pm$ 0.25	1.30 $\pm$ 0.46	3.06 $\pm$ 0.68	2.12 $\pm$ 0.40	26.86 $\pm$ 6.74
Slow (6)	0.54 $\pm$ 0.25	3.09 $\pm$ 0.66	8.75 $\pm$ 0.60	0.97 $\pm$ 0.28	12.62 $\pm$ 2.45

(1) $P < 0.01$; (2) $P < 0.001$; (3) $P < 0.05$; (4) $P < 0.1$ NS (based on *t*-test)

All values $\pm$ s.e. mean for number of patients quoted in brackets

HH = hydralazine hydrazones (acid labile hydralazine); PZ = phthalazinone; TP = *s*-triazolo[3,4-*a*]phthalazine; NAcHPZ = 4-*N*-acetylhydrazinophthalazin-1-one; H = free hydralazine

Table 2 Metabolic ratios for metabolites excreted by patients following twice daily 100 mg doses of hydralazine

Acetylator phenotype	NAcHPZ/HH	NAcHPZ/PZ	TP/HH	PZ/TP
Rapid (7)	22.7 ± 3.66 ¹	12.47 ± 4.38 ²	2.35 ± 0.65 ³	2.71 ± 1.32 ⁴
Slow (6)	4.58 ± 0.92	1.56 ± 0.34	0.34 ± 0.08	15.55 ± 5.09

(1) $P < 0.002$; (2) $P < 0.05$; (3) $P < 0.02$; (4) $P < 0.05$ (based on *t*-test)

All values ± s.e. mean for number of patients quoted in brackets

NAcHPZ = 4-*N*-acetylhydrazinophthalazin-1-one; HH = hydralazine hydrazones (acid labile hydralazine); TP = s-triazolo [3,4-*a*] phthalazine; PZ = phthalazinone

difference in the level of NAcHPZ does not reach statistical significance. Conversely the level of PZ is significantly higher in slow compared with rapid acetylators. Levels of hydralazine hydrazones (acid labile) were, as previously described, higher in slow acetylators (Timbrell *et al.*, 1980). Calculation of the metabolic ratios NAcHPZ/HH, NAcHPZ/PZ, TP/HH and TP/PZ also showed significant differences between phenotypes (Table 2). Free hydralazine, only detected in some of the urines at very low levels, was not included in the calculation of the metabolic ratios. β -glucuronidase treatment had no significant effect on the level of phthalazinone compared with the control incubation.

Discussion

The data presented confirmed by mass spectrometry that PZ, TP and NAcHPZ are urinary metabolites of hydralazine in man as previously reported (Zak, Gilleran, Karliner & Lukas, 1974; Wagner *et al.*, 1977). It can be seen from Table 1 that the excretion of all three metabolites is related to the acetylator phenotype. Thus TP, the terminal product of the primary acetylation pathway, was excreted in significantly greater amounts in the rapid acetylators. Although TP might conceivably arise by formylation (Zak *et al.*, 1974) it is not known whether this reaction shows a phenotypic difference.

The excretion of NAcHPZ was greater in the rapid acetylators although the difference was not statistically significant. This pathway reflects alicyclic oxidation followed by acetylation. As this pathway is of major importance in the metabolism of hydralazine, it is clear that the alicyclic oxidation will be a major determinant of the disposition of hydralazine *in vivo*. The primary product of alicyclic oxidation, HPZ was not detected. This may be due to its instability in aqueous solution, breaking down to PZ and phthalazine-1,4-dione (Facchini, unpublished results).

The excretion of PZ was found to be significantly higher in the slow acetylators compared with rapid acetylators. The origin of this PZ is at present unclear but at least part of that in urine may arise by chemical breakdown of hydralazine (Timbrell, unpublished results) or HPZ (Facchini, unpublished results). The finding that significantly more hydralazine acid-labile hydrazones is excreted by the slow acetylators (Table 1) is consistent with this. Similarly excretion of HPZ would be expected to be greater in slow acetylators, and therefore chemical breakdown of this unstable metabolite would also give rise to more PZ in the slow acetylators. However PZ may also arise directly from hydralazine by oxidative metabolism (Streeter, unpublished results) and could also be produced from phthalazine by the action of aldehyde oxidase (Stubley, Stell & Mathieson, 1979). Any of these routes would be expected to yield more PZ in the slow acetylator phenotype.

The metabolic ratios for these metabolites (Table 2) also show acetylators phenotype differences. The clearest discrimination between phenotypes is shown by the ratio NAcHPZ/HH (Figure 2). Although the other metabolic ratios also show bimodal distributions, there is some overlap of the phenotype. In general however, almost all the subjects fall within the expected phenotype group as determined by the acetylation of sulphamethazine. Although there is no direct correlation between the ratio acetylsulphamethazine/sulphamethazine and any of the metabolic ratios shown in Table 2, there is obviously a correlation between the phenotype as determined by each method (Figure 2). It is clear therefore that hydralazine metabolism is influenced by the acetylator phenotype. The phenotype difference in the amount of TP excreted confirms that the primary acetylation pathway is controlled by the acetylator phenotype. The polymorphic acetylation of HPZ is indicated by the bimodality of the ratio NAcHPZ/HH. The lack of statistical significance for the difference in excretion of this metabolite between phenotypes may reflect the inverse effect of the

Figure 2 Frequency distribution for urinary metabolic ratios: NAcHPZ/HH; TP/HH; NAcHPZ/PZ and PZ/TP for patients of the rapid $\square$ or slow ▨ phenotype TP = s-triazolo [3,4-a]phthalazine; PZ = phthalazinone; NAcHPZ = 4-N-acetylhydrazinophthalazin-1-one; HH = hydralazine hydrazones.

acetylator phenotype on the availability of hydralazine for alicyclic oxidation. However this alicyclic oxidation pathway may be the major pathway and therefore the major determinant of the availability of hydralazine. The increased excretion of PZ in the slow acetylator reflects the use of pathways alternate to acetylation, or possibly the breakdown of hydralazine to HPZ. As previously reported the excretion of hydralazine hydrazones although significantly greater in the slow acetylator, shows only a quantitatively small difference (Timbrell *et al.*, 1980), indicating almost complete metabolism of the drug in both phenotypes.

In patients taking hydralazine the simplest and most easily measurable metabolic ratio is PZ/TP which can be determined in a single urine extract. One patient out of the thirteen studied here would have been wrongly phenotyped if the phenotyping had been performed using this ratio. However, it is possible that this metabolic ratio might, after further study, prove to be a feasible method for acetylator phenotyping hypertensives without interrupting hydralazine therapy.

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