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DETERMINATION OF HYDRALAZINE METABOLITES: 4-HYDRAZINOPHTHALAZIN-1-ONE AND N-ACETYLHYDRAZINOPHTHALAZIN-1-ONE BY GAS CHROMATOGRAPHY AND *s*-TRIAZOLO[3,4-*a*]PHTHALAZINE AND PHTHALAZINONE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

VINCENZO FACCHINI* and JOHN A. TIMBRELL

Clinical Toxicology Unit, Department of Clinical Pharmacology, Royal Postgraduate Medical School, London W12 0HS (Great Britain)

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SUMMARY

Methods are described for the determination of 4-N-acetylhydrazinophthalazin-1-one, 4-hydrazinophthalazin-1-one, phthalazinone and *s*-triazolo[3,4-*a*]phthalazine in human urine.

4-Hydrazinophthalazin-1-one and 4-N-acetylhydrazinophthalazin-1-one (following acid hydrolysis) are reacted with acetylacetone to give a distinctive pyrazole derivative which can be determined by gas chromatography using a nitrogen-specific detector.

Phthalazinone and *s*-triazolo[3,4-*a*]phthalazine are measured underivatised by high-performance liquid chromatography.

INTRODUCTION

Hydralazine (1-hydrazinophthalazine; H) is a potent vasodilating drug, which in combination with a suitable β-blocking drug represents a most effective and widely used antihypertensive regime [1]. However, associated with the use of hydralazine is a lupus erythematosus-like syndrome, an adverse effect almost exclusively confined to the slow acetylator phenotype as determined by the acetylation of sulphamethazine [2].

The metabolism of hydralazine is complex and is known to involve more than one acetylation pathway (Fig.1) [3]. It has recently been shown that one of these acetylation pathways, leading to 3-hydroxymethyltriazolophthalazine (HOMTP) is under the same genetic control as the acetylation of sulphamethazine [4]. However, this metabolic route is not a major pathway, only accounting for 20% of the dose in man. It was therefore important to investi-

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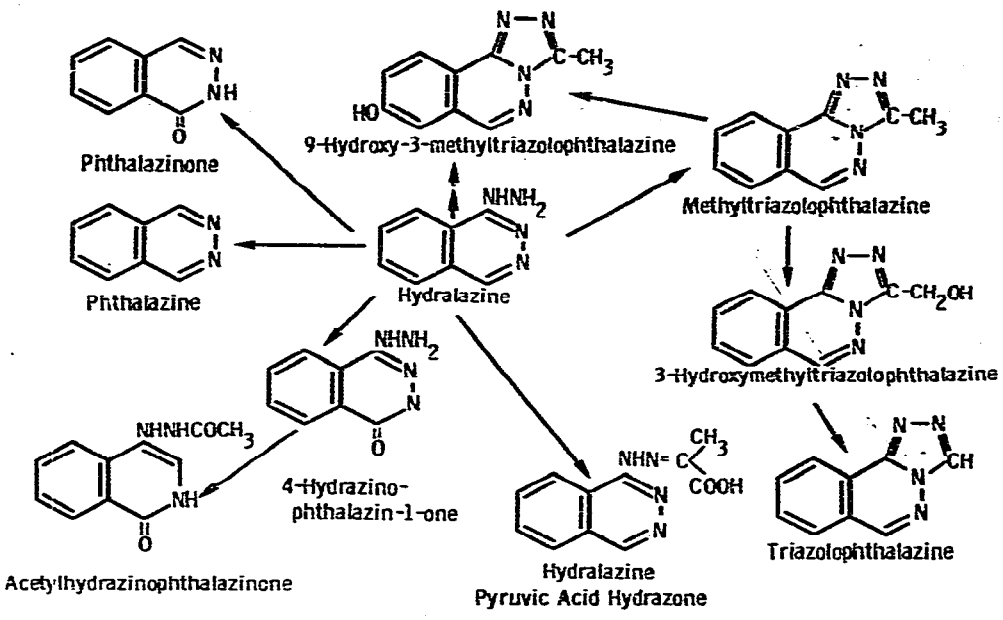

Fig. 1. The metabolism of hydralazine in man.

gate the alternative acetylation pathway, leading to 4-N-acetylhydrazinophthalazin-1-one (NAcHPZ), to define further the effect of the acetylator phenotype on hydralazine metabolism.

It was therefore necessary to devise a method for the measurement of NAcHPZ, and its precursor 4-hydrazinophthalazin-1-one (HPZ). *s*-Triazolo-[3,4-*a*]phthalazine (TP) is the terminal product of the primary acetylation pathway and its measurement is necessary for complete quantitation of this pathway.

As the lupus erythematosus syndrome is confined almost exclusively to slow acetylators, alternative pathways to acetylation are important. One such pathway is the production of phthalazinone (PZ) and a method for quantitation of this metabolite was therefore necessary.

Measurement of these metabolites may also be used for determination of the acetylator phenotype.

EXPERIMENTAL

Chemicals

Hydralazine hydrochloride was obtained from Koch-Light Labs. (Colnbrook, Great Britain); acetylacetone from BDH (Poole, Great Britain). 4-Methylhydralazine (MeH), 4-N-acetylhydrazinophthalazin-1-one, *s*-triazolo[3,4-*a*]-phthalazine, phthalazinone and 4-hydrazinophthalazin-1-one were generously supplied by Ciba-Geigy (Basle, Switzerland).

General methods

Gas chromatography (GC) was carried out on a Perkin-Elmer F17 instrument

fitted with a nitrogen—phosphorus detector. The glass column (2 m × 1.75 mm I.D.) was packed with 10% OV-17 on Gas-Chrom Q, 100—200 mesh (Applied Science Labs., State College, PA, U.S.A.). The detector/injector temperature was set at 300°C and the nitrogen detector used a setting of 6.0 giving a rubidium bead temperature of approximately 600°C. The oven temperature was maintained at 250°C. Nitrogen (carrier gas) flow-rate was set at 35 ml/min.

Gas chromatography—electron impact mass spectrometry (GC—EIMS) was performed on a Finnigan 320 instrument fitted with a 5 ft. × 2 mm I.D. glass column, packed with 10% OV-17 on Gas-Chrom Q, 100—200 mesh, with helium as carrier gas; oven temperature 190°C. An ionisation current of 400 μA and electron energy of 25 eV were used.

High-performance liquid chromatography (HPLC) was carried out using a Waters Model 6000A pump and U6K injector. Reversed-phase chromatography was effected on a column (25 cm × 5 mm I.D.) packed with ODS-Hypersil (octadecyl functional groups bonded to spherical silica particles of 5—7 μm in diameter), supplied by Shandon (Runcorn, Great Britain). The UV detector, a Pye-Unicam LC-UV model, was used at a wavelength setting of 254 nm.

Synthesis of 4-methylphthalazin-1-one

4-Methylphthalazin-1-one (MePZ) used as the internal standard (I.S.) in the determination of PZ and TP, was synthesised in the manner described previously for the preparation of PZ [5]. To 2-acetylbenzoic acid (3.4 g) were added 250 ml of distilled water, 2.06 g of sodium bicarbonate and 4.0 ml of hydrazine hydrate. The mixture was refluxed for 45 min, then acidified by the dropwise addition of concentrated HCl and refluxed for a further 30 min. The hot solution was then filtered and evaporated, and the residue extracted with five 50-ml aliquots of boiling chloroform. The pooled filtered extracts were then filtered and evaporated to leave a white powder which was recrystallised from chloroform. GC—EIMS gave a single peak which yielded the following mass spectrum: *m/e* 160 (M^+ parent ion and base peak); 132 (M−28; M−CO); 131 (M−29); 105 (M−55); 104 (M−56); 103 (M−57). This analysis revealed a molecular ion and fragmentation pattern consistent with the structure of MePZ.

Assay procedures

4-Hydrazinophthalazin-1-one. As the determination of NAcHPZ (see below) involves hydrolysis to HPZ, any free HPZ existing in the urine sample prior to hydrolysis, will also be determined. The assay for HPZ is based on derivatisation with acetylacetone to form a pyrazole which can be assayed by GC as previously described [6,7].

Duplicate urine samples (10 ml) were adjusted to pH 3 (at this pH there is no hydrolysis of NAcHPZ to HPZ) and MeH (I.S.) 0.04 mg added. After reaction with excess acetylacetone (0.5 ml) for 1 h at room temperature the urine was adjusted to pH 9.5 and extracted with methylene chloride (30 ml). The extract was filtered through phase separating paper, the filtrate evaporated to dryness and the residue dissolved in ethyl acetate (1.0 ml). Aliquots (1—5 μl) of the ethyl acetate solution were then injected into the gas chromatograph as described above. The ratio of the peak heights of HPZ to MeH (I.S.) derivatives was determined.

A standard curve was constructed by spiking blank urine samples with HPZ and MeH, reacting with acetylacetone at pH 3, extracting and analysing the extracts as described above.

4-N-Acetylhydrazinophthalazin-1-one (NAcHPZ). This metabolite was determined after acid hydrolysis to yield HPZ which is simultaneously reacted with acetylacetone to form a pyrazole derivative as described above (Fig. 2).

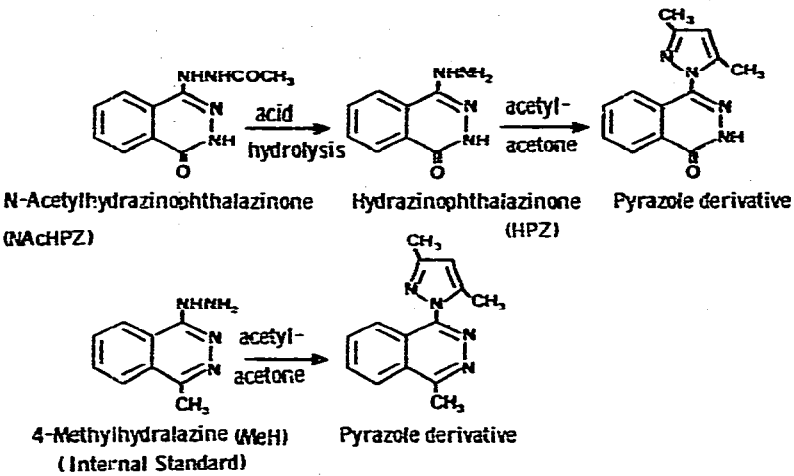

Fig. 2. Structure of hydralazine metabolites, internal standard and their respective pyrazole derivatives necessary for GC.

To duplicate urine samples (10 ml) were added MeH (I.S.) (0.05 mg), excess acetylacetone (0.5 ml) followed immediately by concentrated hydrochloric acid (5.0 ml) with immediate vortexing to ensure adequate mixing. After reaction for 2 h at room temperature with periodic vortexing the samples were adjusted to pH 9.5 and processed as described for the determination of HPZ.

The ratio of the peak heights HPZ to MeH were determined by GC using the conditions described above.

A standard curve was constructed by spiking blank urine samples with NAcHPZ and MeH (I.S.) and assaying as described above.

s-Triazolo[3,4-a]phthalazine (TP) and phthalazinone (PZ). These metabolites were determined underivatised by HPLC.

To duplicate urine samples (10 ml) MePZ (I.S.) (0.1 mg) was added and the pH adjusted to pH 9.5. The samples were then extracted with methylene chloride (30 ml), the extract filtered through phase separating paper and then reduced to dryness. The residue was dissolved in methanol (1.0 ml) and was assayed by HPLC. Aliquots (1.5 µl) of this extract were injected into the instrument and eluted with methanol—water (15:85), 1.8 ml/min. PZ, TP and MePZ (I.S.) were detected by UV absorption at 254 nm and the ratio of the peak heights of PZ/MePZ and TP/MePZ were determined. Standard curves were constructed by spiking blank urine with known amounts of PZ, TP and MePZ (I.S.).

RESULTS AND DISCUSSION

The methods described for the determination of NAcHPZ, HPZ, TP and PZ are straightforward and specific. Adequate separation is achieved between each metabolite under investigation and the respective internal standards, and no interfering peaks are present in blank urine (Fig. 3).

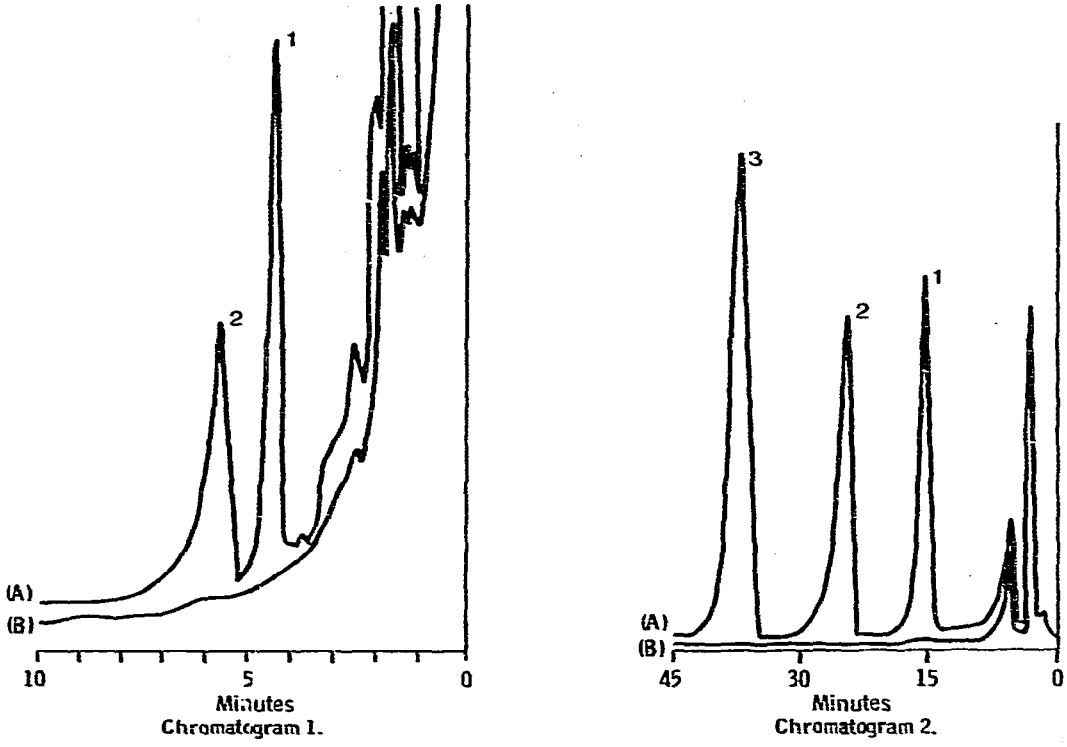

Fig. 3. Chromatogram 1: (A) extract of urine containing NAcHPZ or HPZ and MeH (I.S.). Peaks: 1 = 1-(3',5'-dimethylpyrazole)4-methylphthalazine (I.S.); 2 = 1-(3',5'-dimethylpyrazole)phthalazin-4-one; (B) extract of blank urine. Chromatogram 2: (A) extract of urine containing PZ (peak 1), TP (peak 2) and MePZ (I.S.) (peak 3); (B) extract of blank urine.

The standard curves, prepared by spiking blank urine with various known amounts of the compounds in question and the respective internal standards, are linear over the concentration ranges assayed. The curves were fitted by regression analysis, and parameters of accuracy and sensitivity are shown in Table I. Preliminary studies of the urine from patients given hydralazine have indicated that the methods described are suitable for the determination of these metabolites.

TABLE I

ACCURACY AND SENSITIVITY OF THE ASSAY METHODS

Compound	Concentration range of standard curve (μg/ml)	Regression coefficient	Standard error* (μg/ml)	Slope** ± S.E.	Intercept** ± S.E.	Sensitivity (μg/ml)
HPLC assay						
Phthalazinone	2–15	1.0	(2) ± 0.10	15.64 ± 0.80	−0.02 ± 0.01	0.5
s-Triazolo[3,4-a]phthalazine	2–15	0.99	(2) ± 0.25	12.89 ± 0.40	−0.03 ± 0.03	0.5
GC assay						
4-Hydrazinophthalazin-1-one	1–6	0.99	(1) ± 0.01	27.12 ± 1.89	−0.05 ± 0.07	0.25
4-N-Acetylhydrazinophthalazin-1-one	4–20	1.0	(4) ± 0.15	8.86 ± 0.27	−0.09 ± 0.03	1.0

*Standard error calculated for one point on the curve (4 determinations), concentration in brackets.
**Standard error of the slope and intercept calculated from the five points of the calibration curve by linear regression.

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