Journal of Chromatography, 187 (1980) 218–223

CHROM. 12,318

Note

Determination of hydralazine and its acetylated metabolites in urine by gas chromatography and high-pressure liquid chromatography

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(Received August 16th, 1979)

Hydralazine is becoming increasingly widely used as an anti-hypertensive drug particularly in combination with other anti-hypertensives such as propranolol[1]. The use of hydralazine in man is attended by a significant incidence of a lupus erythematosus-like syndrome[2]. This adverse effect occurs almost exclusively in the slow acetylator phenotype, as determined by the sulphamethazine test. This and other evidence has suggested that hydralazine is acetylated in man and that this acetylation is under the same genetic control as that for sulphamethazine[3].

Although the metabolism of hydralazine has been studied in man[4,5] and a number of methods have been devised for measurement of hydralazine in plasma and urine[5–7], methods for measurement of methyltriazolophthalazine (MTP) and hydroxymethyltriazolophthalazine (3-OHMTP) suitable for the analysis of large numbers of urine samples have not been described. Consequently it was of interest to devise methods for measuring hydralazine and its acetylated metabolites in urine to show whether hydralazine is indeed polymorphically acetylated in man. Furthermore it would also provide a means of acetylator phenotyping patients taking hydralazine, without stopping their therapy, as would otherwise be necessary if the sulphamethazine test is used.

EXPERIMENTAL

Reagents

Hydralazine hydrochloride was obtained from Koch-Light Labs. (Colnbrook, Great Britain); acetylacetone from BDH (Poole, Great Britain) and bis-trimethylsilyl(TMS)acetamide, 3-hydroxypropionic acid and trifluoroacetic anhydride (TFA) from Aldrich (Gillingham, Great Britain.).

β-Glucuronidase, type 2 (Helix pomatia) containing sulphatase activity was supplied by Sigma, (Kingston, Great Britain).

Methylhydralazine, MTP and 3-OHMTP 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 column, 2 m × 1.75 mm I.D. glass 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° and the nitrogen detector used at a setting of 6.0, giving a rubidium bead temperature of approximately 600°.

High-pressure liquid chromatography (HPLC) was carried out using a Waters Model 600A pump and U6K injector. The column, 25 cm × 5 mm I.D., was packed with Hypersil ODS (Shandon, Runcorn, Great Britain). The UV detector, a Pye-Unicam Model LC UV was used at a wavelength setting of 254 nm.

GC–electron impact mass spectrometry (EI-MS) was performed on a Finnigan 320 instrument. The instrument was fitted with a 5 ft. × 2 mm I.D. glass column, packed with 10% OV-17 on Gas-Chrom Q, 100–120 mesh, with helium as carrier gas. An emission current of 500 μA and electron energy of 25 eV were used.

Syntheses

Trifluoromethyltriazolophthalazine (F_3-MTP). This compound, used as an internal standard (I.S.), was prepared in the manner described for the preparation of MTP[8]. Hydralazine hydrochloride was refluxed with excess TFA for 4 h. The reaction mixture was reduced to dryness under vacuum and dissolved in hot ethyl acetate. The hot ethyl acetate solution was filtered and the filtrate decolorised with charcoal. After filtration of the solution to remove the charcoal, the product was allowed to crystallise. Analysis by GC revealed a single peak, retention time 2.1 min at 250°. GC–EI-MS similarly revealed a single peak, giving *m/e*:238 (M^+); 141, 129, 128, 115 (M—123; base peak).

3-Hydroxyethyltriazolophthalazine (3-OHETP). This compound, used as I.S., was synthesised in a similar manner to 3-OHMTP[9]. Hydralazine hydrochloride was refluxed at 100° with excess 70% aq. hydroxypropionic acid for 14 h. After refluxing the reaction mixture was washed into a separating funnel with chloroform, neutralised with excess sodium bicarbonate solution and then filtered through phase-separating paper and the volume reduced. On addition of toluene a solid precipitated. After filtering off the solid was twice crystallised from boiling toluene and the pale cream solid further recrystallised from ethylacetate. After silylation with bis-TMS-acetamide the compound gave a single peak on GC at 270° with a retention time 6.8 min. GC–EI-MS of the silyl derivative similarly revealed a single peak, giving *m/e*: 286 (M^+); 285 (M—1); 271; 255; 196; 73 (M—213; base peak).

Hydralazine pyruvic acid hydrazone. This metabolite of hydralazine was prepared by reacting equimolar amounts of hydralazine hydrochloride and sodium pyruvate at 30°, pH 5, in water. The hydralazine sodium pyruvate hydrazone precipitated and was filtered off. The yellow solid was dissolved in water at pH 7.0 and the aqueous solution extracted three times with methylene chloride. The aqueous solution was adjusted to pH 4, the pyruvic acid hydrazone precipitated and was filtered off and dried in a dessicator. GC of the hydrazone as a solution in ethyl acetate yielded a single peak, retention time 3.9 min (250°). This peak was found to have an identical retention time to methyltriazolophthalazine. GC–EI-MS of the hydrazone yielded one peak which gave the mass spectrum of methyltriazolophthalazine: 184 (M^+, base peak), 156 (M—28); 155 (M—29), 115 (M—69). GC–EI-MS of the hydrazone methylated "on-column" with trimethylanilinium hydroxide yielded a

single peak, retention time 9.4 min. (240°, 10% OV-17), *m/e*: 258 (M^+), 199 (M—59, base peak), 158 (M—100) 146, 144, 143, 131, 117.

Assay procedures

Hydralazine. The assay for hydralazine was based on derivatisation with acetylacetone to form a pyrazole which could be assayed by GC as described by Smith *et al.*[7]. However, it was discovered that MTP, one of the metabolites of hydralazine, had an identical retention time to the hydralazine pyrazole derivative on GC. Therefore, it was necessary to remove this MTP by pre-extraction of urine. Hydralazine in urine will be present mainly in the form of hydrazones with α-keto acids. The pyruvate hydrazone of hydralazine was found to be extracted at acid pH, therefore for removal of MTP, extraction at an alkaline pH (pH 9.5) was necessary.

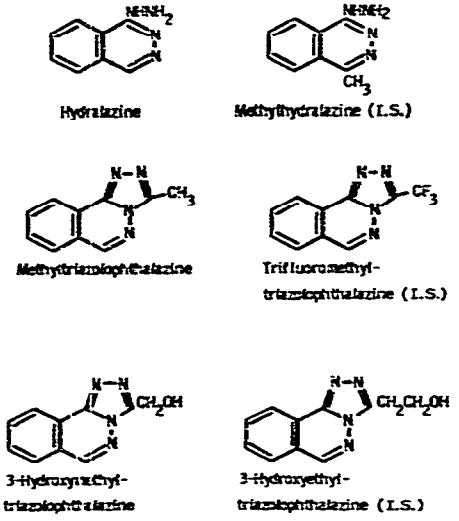

Fig. 1. Metabolites and internal standards measured.

Duplicate urine samples (10 ml) were adjusted to pH 9.5 and extracted with methylene chloride (1 × 30 ml). The extracted urine was adjusted to pH 0.1 and the I.S., methylhydralazine was added. After reaction with excess acetylacetone for 1 h at room temperature the urine was readjusted to pH 9.5 and extracted with methylene chloride (1 × 30 ml). The extract, filtered through phase-separating paper, was then reduced to dryness and the residue dissolved in ethylacetate (1.0 ml). Aliquots of the ethyl acetate solution were injected into the gas chromatograph (10% OV-17, 250°, nitrogen flow-rate 35 ml/min), fitted with a nitrogen specific detector.

The ratio of the peak heights of the hydralazine (HP) and methylhydralazine derivatives (MeHP) was determined. A standard curve was constructed by spiking blank urine samples with hydralazine hydrochloride, and internal standard, reacting with acetylacetone at pH 0.1 and extracting as described above. Also a known amount of synthetic pyruvic acid hydrazone was added to blank urine and carried through the assay.

MTP. This metabolite (Fig. 1) was measured underivatised by HPLC. To duplicate urine samples (10 ml), F_3-MTP, the I.S. was added and the pH adjusted to 9.5. The urine was then extracted with methylene chloride (1 × 30 ml), the extract filtered through phase-separation paper and then reduced to dryness. The residue, taken up in methanol (1.0 ml) was assayed by HPLC. Aliquots (1–5 μl) were injected into the chromatograph and eluted with methanol–water (60:40), 1.5 ml/min. MTP and the I.S. were detected by UV absorption at 254 nm and the ratio of peak heights determined. A standard curve was constructed by spiking blank urine with known amounts of MTP.

3-OHMTP. This metabolite (Fig. 1) is present in urine mainly in the form of conjugates which must be hydrolysed with β-glucuronidase/sulphatase before the assay.

Duplicate urine samples (10 ml) were adjusted to pH 5 with acetate buffer (0.01 *M*) (10 ml) and incubated with β-glucuronidase/sulphatase for 24 h at 37°. After the incubation, the I.S., 3-OHETP was added, the urine adjusted to pH 2.5 and extracted with methylene chloride (1 × 30 ml). The extract, filtered through phase-separating paper, was reduced to dryness and silylated with bis-TMS-acetamide at 60–80° for 1 h. The excess silylating agent was removed under nitrogen and the residue taken up in ethyl acetate (1.0 ml). Aliquots were injected into the gas chromatograph fitted with a nitrogen specific detector, oven temperature 270°, carrier-gas flow-rate 35 ml/min.

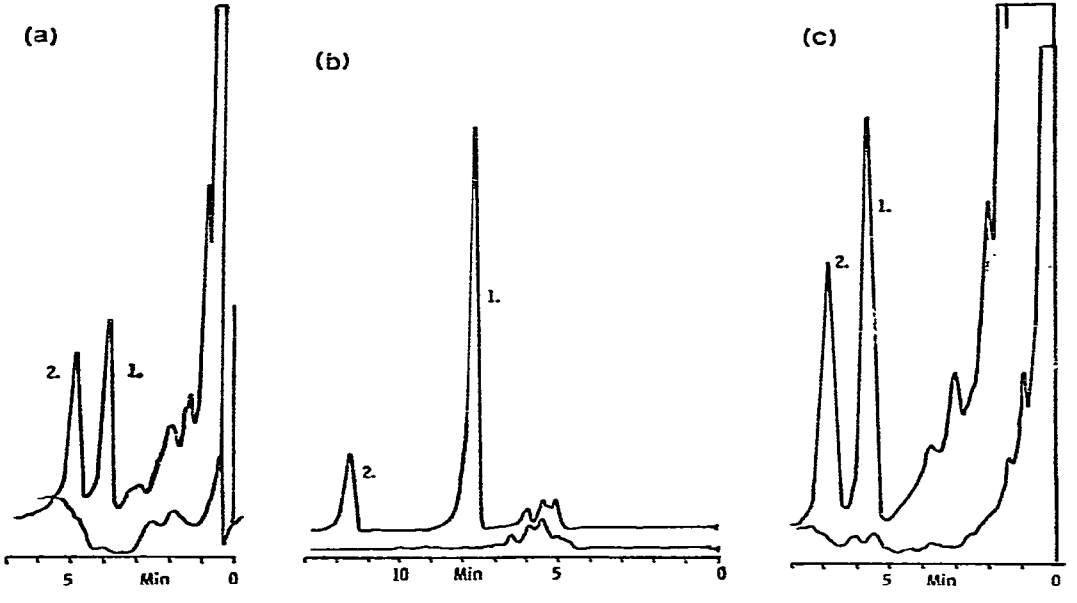

Fig. 2. (a) Chromatogram of extract of urine containing hydralazine. Peaks: 1 = 1-(3′,5′-dimethylpyrazole)phthalazine; 2 = 1-(3′,5′-dimethylpyrazole) 4-methylphthalazine (I.S.). (b) Chromatogram of extract of urine containing MTP. Peaks: 1 = MTP; 2 = F_3-MTP (I.S.). (c) Chromatogram of extract of urine contianing 3-OHMTP. Peaks: 1 = 3-OHMTP-TMS derivative; 2 = 3-OHETP-TMS derivative (I.S.). Lower traces are chromatograms of blank urine extracts.

The peak heights of the 3-OHMTP-TMS derivative and I.S.-TMS derivative were measured and the ratio computed. A standard curve was prepared by spiking blank urine with known amounts of standards.

RESULTS AND DISCUSSION

The methods described for the determination of hydralazine, MTP and 3-OHMTP are relatively straightforward and specific.

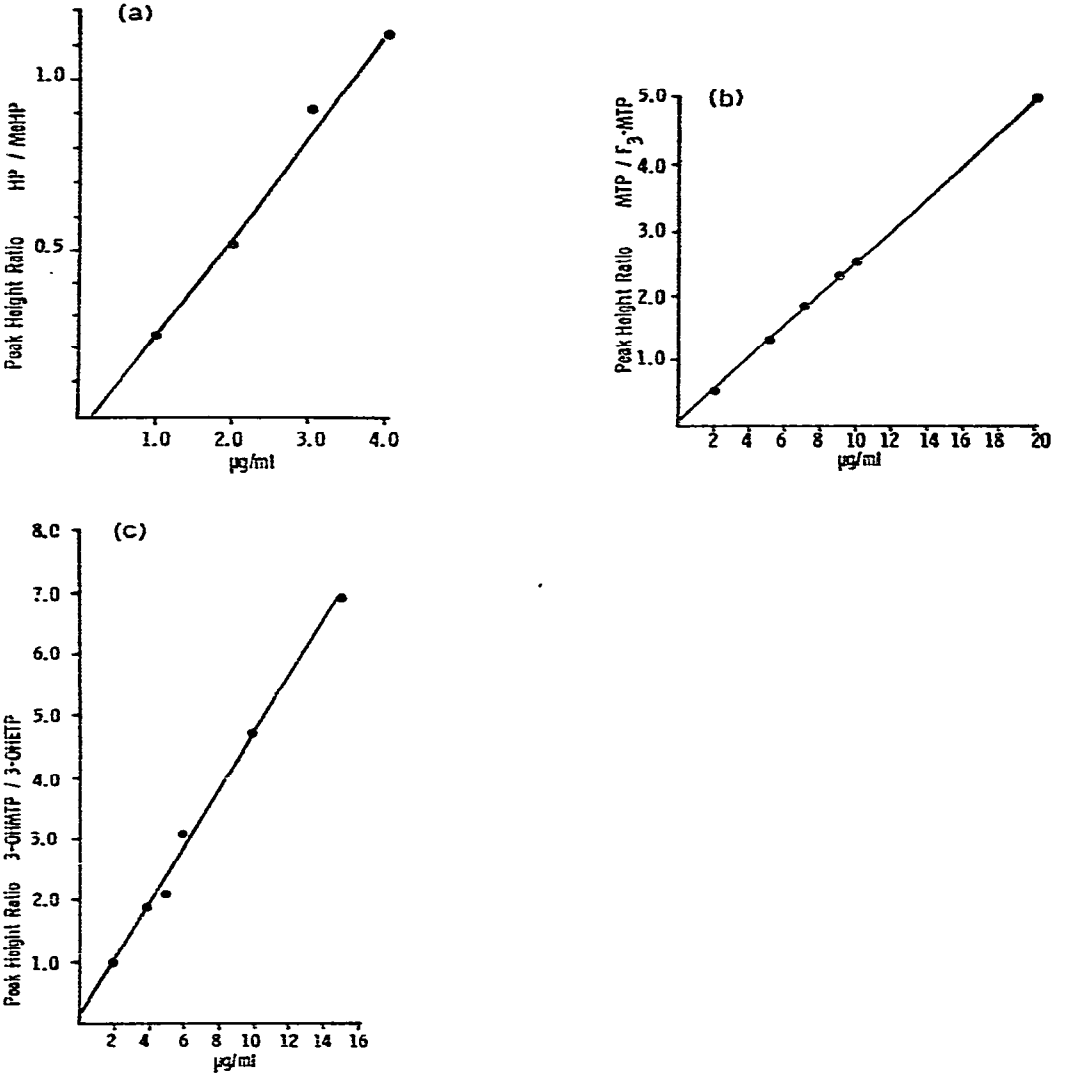

Fig. 3. Calibration curves for measurement of metabolites in urine. (a) Hydralazine. (b) MTP. (c) 3-OHMTP. Peak height ratio (metabolite/I.S.) plotted against concentration. Standard curves were prepared by spiking urine with metabolites and I.S. Each point is the mean of at least two determinations in duplicate.

As can be seen from the chromatograms in Fig. 2 adequate separation is achieved between each derivative and I.S. to permit quantitation and no interfering peaks are present in blank urine.

The standard curves, prepared by spiking blank urine with various known amounts of the compounds in question and I.S., are shown in Fig. 3. They are linear over the concentration ranges assayed. The standard curves were fitted by regression analysis and the regression coefficients and accuracy of the method are shown in Table I. Studies of urine from patients treated with hydralazine have indicated that the methods described are suitable for the determination of these metabolites which are within the concentration ranges of the standard curves[10]. Methods are being devised for the determination of hydralazine in the presence of MTP so that free hydralazine as well as the pyruvate hydrazone can be determined.

TABLE I

ACCURACY AND SENSITIVITY OF THE ASSAY METHODS

Compound	*Conc. range of standard curve (μg/ml)*	*Regression coefficient (r^2)*	*Standard deviation (μg/ml)**	*Sensitivity (μg/ml)*
Hydralazine	1–5	0.98	±0.04 (1)	0.5
Methyltriazolophthalazine	2–20	1.00	±0.06 (5)	0.5
3-Hydroxymethyltriazolophthalazine	2–15	0.99	±0.05 (4)	0.5

* Standard deviation calculated for one point on curve, concentration in brackets.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Roy Clare for running the mass spectra, Miss Ann Tolman for typing the manuscript and Mr. Stephen Sutton for his expert technical assistance.

This work was supported by grants from the Nuffield Foundation and Ciba-Geigy, one of us (A.J.S.) was supported by a Medical Research Council Research Studentship.

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