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GAS CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF HYDRALAZINE AND ITS ACETYLATED METABOLITE IN SERUM USING A NITROGEN-SELECTIVE DETECTOR

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SUMMARY

A relatively simple gas chromatographic method has been developed for the quantitative determination of hydralazine simultaneously with its acetylated metabolite, 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP). The proteins were removed by means of sulfosalicylic acid and Sure-Sep®. On treatment with formic acid, hydralazine and its internal standard were converted into their formylated derivatives. These derivatives, MTP and its internal standard were extracted with toluene and determined by gas chromatography with a nitrogen-selective detector. The lower limits of detection for hydralazine and MTP were 0.13 and 0.27 μmol/l, respectively.

INTRODUCTION

N-Acetylation seems to be one of the major metabolic pathways for hydralazine in man [1]. Measurement of both hydralazine and the acetylated metabolite, 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP) is therefore of interest.

Since hydralazine is not extractable from biological material with organic solvents a method based on derivatization directly in the biological milieu is needed. Many papers have been published on the measurement of hydralazine, but only a recently published gas chromatographic (GC) method has sufficient specificity and sensitivity. In this GC method hydralazine is converted into tetrazolo[1,5-α]phthalazine and detected by an electron-

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capture detector [2]. A slight modification of this procedure allows simultaneous determination of MTP [3].

We present a GC method using a nitrogen-selective detector for the simultaneous determination of hydralazine and MTP. Hydralazine is analysed as its formylated derivative, *s*-triazolo[3,4-α]phthalazine (TP) (see Fig. 1), and detected by a nitrogen-selective detector. The described method is relatively simple and therefore well suited for the routine determination of hydralazine in serum from patients under treatment with this drug.

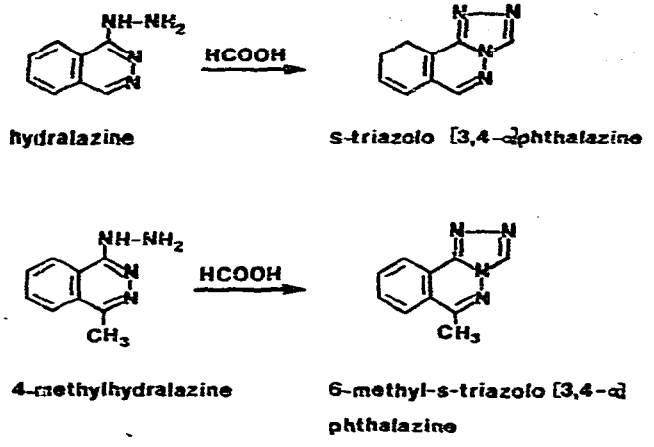

Fig. 1. Transformation of hydralazine and 4-methylhydralazine (internal standard) to their formyl derivatives.

EXPERIMENTAL

Materials

Hydralazine hydrochloride, 4-methylhydralazine hydrochloride (internal standard) and 3-methyl-*s*-triazolo[3,4-α]phthalazine (MTP, Fig. 2) were a gift from Ciba-Geigy (Basle, Switserland). 3-Ethyl-*s*-triazolo[3,4-α]phthalazine [ETP (Fig. 2), internal standard] was synthesized as described for the analogous methyl derivative by Haegele et al. [1].

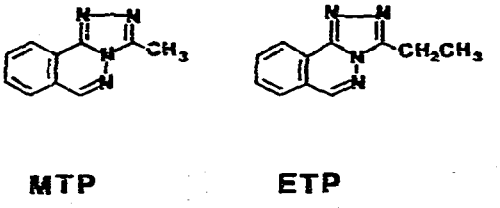

Fig. 2. Structural formulae of the acetylated metabolite of hydralazine (MTP) and its internal standard (ETP).

Serum standards were prepared every day of analysis and 10% ascorbic acid solution added as described below for the serum samples. Stock solutions of hydralazine hydrochloride (0.5 mmol/l of 0.1 *N* HCl) and 4-methylhydralazine hydrochloride (1.7 mmol/l of 0.1 *N* HCl) were kept at 2—8°C for two and four weeks, respectively. Stock solutions of MTP and ETP (1.3 mmol/l of ethanol) were kept at −20°C for several months. All reagents were of analytical quality. Sure-Sep® (plasma/serum separators) was purchased from General Diagnostics (Morris Plains, NJ, U.S.A.).

Serum samples

Within 15 min of blood collection the serum was separated, 5 μl of 10% ascorbic acid solution per ml of serum were added and the samples stored at −20°C.

Procedure

To 1.0 ml serum were added 100 μl of 4-methylhydralazine solution (16.6 μmol/l), 125 μl of ETP solution (12.6 μmol/l) and 100 μl of 50% sulfosalicylic acid solution. The reagents were whirlimixed for 5 sec and centrifuged (5 min at 1300 *g*). After adding Sure-Sep® the centrifugation was repeated. The supernatant was decanted, 100 μl of formic acid were added, then the test-tube was sealed and placed at 100°C for 20 min. After cooling at room temperature (about 3 min), 400 μl of 5 *M* NaOH, 1.5 ml of 1 *M* carbonate buffer (pH 10.5) and 3 ml of toluene were added. The mixture was shaken for 5 min, centrifuged for 5 min (1300 *g*) and the toluene phase transferred to a new test-tube. The extraction procedure was repeated and the combined toluene extracts were evaporated under nitrogen at 50°C. The evaporated samples were stored at −20°C until the GC analysis could be carried out.

The dry residue was dissolved in 30 μl of toluene and 2 μl were analysed by GC. The serum concentrations of hydralazine and MTP were calculated on the basis of the peak-height ratios of hydralazine/4-methylhydralazine and MTP/ETP by reference to the graphs obtained by analysing serum standards simultaneously.

Gas chromatography

A Model 5830A gas chromatograph equipped with a nitrogen-selective detector (Hewlett Packard, Palo Alto, CA, U.S.A.) was used with the following operating conditions: a 2.0 × 2 mm I.D. glass column was packed with 1% SP 1000 on Chromosorb W 80—100 mesh; the carrier gas (helium) flow-rate was 30 ml/min, the air flow-rate 50 ml/min and the hydrogen flow-rate 3 ml/min; injector temperature was 220°C, detector temperature 300°C. The oven was programmed from 220 to 250°C at 16°C/min. The voltage of the rubidium bead was set at 16—20 V.

RESULTS

Evaluation of the analytical procedure

Fig. 3 shows chromatograms obtained from serum analysis. Plots of the standard curve of hydralazine over the range 1—15 μmol/l were linear and

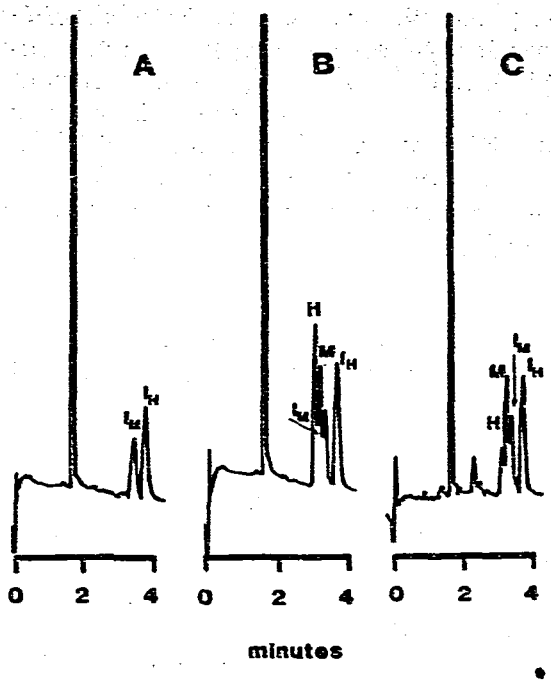

Fig.3. Chromatograms of human serum extracts, analysed as described in the text. A, Serum; B, serum to which hydralazine and MTP were added (2.5 and 1.2 μmol/l, respectively); C, serum from a patient, therapeutically treated with hydralazine. H = Hydralazine, M = MTP, I_M = internal standard for MTP, I_H = internal standard for hydralazine.

passed through the origin. The standard curve of MTP was linear over the same range but did not pass through the origin. The blank was from 0.05 to 0.15 μmol/l.

The lower limits of detection for hydralazine and MTP were 0.13 and 0.27

TABLE I

REPRODUCIBILITY OF REPLICATE ANALYSES OF HYDRALAZINE AND MTP ADDED TO HUMAN SERUM

The serum samples were stored at −20°C from one day to seven weeks and analysed at random on different days.

Compound	Serum concentration* (μmol/l)	Coefficient of variation (%)
Hydralazine	9.92	4.9
	3.30	3.4
	0.50	7.7
MTP	14.64	6.4
	4.86	4.8
	0.88	9.4

*Mean values from eight duplicate samples of each concentration.

TABLE II

STABILITY OF HYDRALAZINE AND MTP IN SERUM SAMPLES AFTER STORAGE AT −20°C

	Hydralazine (μmol/l)				MTP (μmol/l)			
	1st determination	2nd determination	Time between 1st and 2nd determination	Deviation (%)	1st determination	2nd determination	Time between 1st and 2nd determination	Deviation (%)
Patient samples	1.17	1.11	6 weeks	−5.1	< 0.27	< 0.27	6 weeks	—
Patient samples	1.32	1.34	6 weeks	+1.5	0.31	0.34	6 weeks	+9.7
Patient samples	3.28	3.46	6 weeks	+3.1	0.53	0.54	6 weeks	+1.9
Spiked samples	2.38	2.60	6 months	+9.2	1.24	1.25	6 months	+0.8
Spiked samples	4.87	4.98	7 months	+2.2	2.53	2.47	7 months	−2.4

μmol/l, respectively (25 and 50 μg/l). The reproducibility was determined using spiked serum samples analysed at random on different days. These spiked serum samples were stored at −20°C from one day to seven weeks. The reasonable reproducibility shown in Table I demonstrates that hydralazine and MTP are stable for weeks under the conditions mentioned above. The stability of hydralazine at −20°C is confirmed by the results in Table II, where both patient serum samples and spiked samples were determined twice with a time interval of from six weeks to seven months.

Table III compares results from analyses of patient serum samples by our GC method and another GC method [4].

Application of the analytical procedure

Venous blood samples from 32 patients, therapeutically treated with 50—200 mg (0.25—1.0 mmol) of hydralazine per day, were collected just before and 1 h after the first morning dose.

The ranges of the serum levels of hydralazine were found to be <0.13—7.6 and 0.50—12.7 μmol/l, respectively (<25—1500 and 100—2500 μg/l).

The MTP levels were <27 and <0.27—8.2 μmol/l, respectively (<50 and <50—1500 ng/ml).

TABLE III

COMPARISON OF RESULTS OF ANALYSIS OF PATIENT SERUM SAMPLES BY OUR GC METHOD AND ANOTHER GC METHOD [4]

Sample No.	Our results	Results from another GC method [4]
	Hydralazine concentration (μmol/l)	"Apparent" hydralazine concentration (μmol/l)
1	4.6	5.3
2	5.5	3.9
3	1.9	2.9
4	4.8	3.2
5	13.1	9.2
6	0.6	0.2

DISCUSSION

Several authors have mentioned the instability of hydralazine in human serum during storage [2,5]. In consequence, Jack et al. [2] recommend derivatization before cold storage. We found that, after the addition of ascorbic acid, serum samples were stable at −20°C for at least seven months (Table II). Furthermore, we have discovered that the ascorbic acid prevents cleavage of hydralazine in the analytical procedure. The use of sulfosalicylic acid as denaturating agent and separation of the denaturated proteins by means of Sure-Sep® have been shown to remove the proteins so completely, that no interfering peaks appeared in the chromatogram.

We have tested many derivatives of hydralazine such as tetrazolo[1,5-α]-phthalazine [2] and formaldehyde, acetaldehyde and acetone hydrazones of hydrazine, but we found neither as sensitive at the nitrogen-sensitive detector as the formyl derivative of hydralazine (TP).

The yield of the derivatization was found to be about 50% by comparison with TP, prepared on an analytical scale as described by Haegele et al. [6]. Different reaction conditions have been studied to optimize the derivatization of hydralazine. An elevated temperature was necessary; 100°C was chosen to complete the reaction within 10—20 min. Neither a longer reaction time (60 min) nor more formic acid (500 μl) improved the yield. Both TP and MTP appeared to be rather stable. No loss of TP or MTP was observed in evaporated samples, neither after standing at 50°C for 15 min nor after storage for one week at −20°C. The yield of one extraction with toluene was only about 50%. Consequently, two extractions raised the yield to about 75%.

SP 1000 turned out to be the best stationary phase since phases as OV-17 and SE-30 resulted in extreme tailing. Although the peaks are rather close (Fig. 3), the separation has proved satisfactory to obtain a reasonable precision (Table I). A column 3 m long improved the separation, but not the precision.

Earlier published GC methods [2,3] used an electron-capture detector, but recently a method using a nitrogen-selective detector has been published [7]. It is our opinion that a nitrogen-sensitive detector is more suited for routine analysis. In our laboratory nitrogen-sensitive detectors have been used without problems by different technicians during the past four years.

TP has been identified as a metabolite in rat urine [1] and recently in human urine, too [6]. But like Haegele et al. [6] we did not find any detectable amount in the serum from patients therapeutically treated with hydralazine.

Some authors have postulated the existence of some acid-hydrolysable conjugates of hydralazine [4,7,8]. These findings are confirmed by Haegele et al. [6], who identified acetone, pyruvate, and α-ketoglutarate hydrazones. In vitro activity studies, however, have shown that these hydrazones are at least as active as parent hydralazine [6,9]. During the last few years it has been shown [4,7,8] that earlier published methods [2,5] involving acidic treatment of the samples do not distinguish between hydralazine and its acid-hydrolysable conjugates, but recently Degen [7] has described a GC method for the specific determination of unchanged hydralazine.

In view of the acid conditions in our derivation procedure (pH <1) these conjugates are most likely co-determined in our analysis. A comparison of our method with another GC method (Table III) indicates that this actually is the case, because our results are in reasonable agreement with the "apparent" hydralazine concentrations (hydralazine with co-determination of the acid-hydrolysable conjugates).

Great individual variations in serum concentrations of hydralazine and MTP were found in patients therapeutically treated with almost the same dose of hydralazine. It has already been shown that the different extent of acetylation of hydralazine in man [3,10] is an important determinant of the

plasma level of hydralazine, but many other factors may equally be the causes of the great variations in bioavailability of hydralazine.

Earlier investigators [10] have shown that the hypotensive effect of hydralazine is proportional to its plasma concentration. Our preliminary results from blood pressure measurements and serum concentration determinations seem to show correlation, too [1]. This indicates that the described method may be a useful guide to a more rational and safe dosage of hydralazine.

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