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DETERMINATION OF UNCHANGED HYDRALAZINE IN PLASMA BY GAS–LIQUID CHROMATOGRAPHY USING NITROGEN-SPECIFIC DETECTION

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SUMMARY

A gas chromatographic method is described for the specific determination of unchanged hydralazine in plasma. On treatment with 2,4-pentanedione, hydralazine is converted into 1-(3,5-dimethyl-1-pyrazolyl)phthalazine, a stable compound which is easily extracted from biological material and determined quantitatively by gas chromatography with nitrogen-specific detection. 4-Methylhydralazine is used as an internal standard. The sensitivity of the method is *ca.* 10 ng/ml. Hydrazones of hydralazine do not interfere with the assay.

INTRODUCTION

A number of analytical procedures have been developed for the determination of hydralazine (1-hydrazinophthalazine, active ingredient of Apresoline®) in biological material: (1) Perry[1], 1953 (ninhydrin complex formation and spectrophotometry); (2) Schulert[2], 1961 (*p*-hydroxybenzaldehyde hydrazone formation and spectrophotometry); (3) Zacest and Koch-Weser[3], 1972 (modification of procedure 2); (4) Zak *et al.*[4], 1974 (*p*-methoxybenzaldehyde hydrazone formation and spectrophotometry); (5) Jack *et al.*[5], 1975 (tetrazolophthalazine formation and gas chromatography); (6) Talseth[6], 1976 (modification of procedure 5); (7) Haegele *et al.*[7], 1976 (modification of procedure 6); (8) Zak *et al.*[8], 1977 (modification of procedure 7); (9) Haegele *et al.*[9], 1977 (cyclohexanone hydrazone formation and gas chromatography–mass spectrometry).

However, almost all of these methods measure both unchanged hydralazine and its hydrazone derivatives. Moreover, the hydrazones present in the plasma are susceptible to hydrolysis at low pH and regenerate hydralazine[10].

The first gas chromatographic (GC) method published[5], which is based on the formation of a tetrazolophthalazine, does not differentiate between "free" and "bound" hydralazine. It was therefore modified by Zak *et al.*[8]. In the modified procedure a pH range of 2.85–3.90 is employed to ensure derivatization of only the free hydralazine. Following oxidative degradation of the free hydralazine with

MnO_2, the hydrazones are hydrolyzed and derivatized at pH 0.9 in the presence of nitric acid.

In a recent study by Schneck *et al.*[11] free hydralazine was estimated from the difference between assays of acid-labile conjugates and of total hydralazine. This procedure was applied to plasma samples from subjects of slow or fast acetylator phenotype receiving repeated doses of hydralazine. The levels of free hydralazine reported were considerably higher than those of the acid-labile hydrazones. Since this finding is in conflict with previously reported data found in single dose experiments, where the free hydralazine was measured directly[8,12], it is necessary to confirm the pharmacokinetics of free hydralazine in human plasma by use of chemical analytical principles different from those employed to date.

A procedure previously reported by Smith *et al.*[13] for the specific determination of unchanged hydralazine in Apresoline® tablets was therefore modified for its application to biological samples. The assay is based on the formation of 1-(3,5-dimethyl-1-pyrazolyl)phthalazine after addition of 2,4-pentanedione directly to plasma (Fig. 1). This method has several advantages over all previous ones. First, the reaction can be carried out under conditions which do not affect the labile hydrazones. Secondly, the derivative formed has favourable properties for GC and detection by a nitrogen-specific detector.

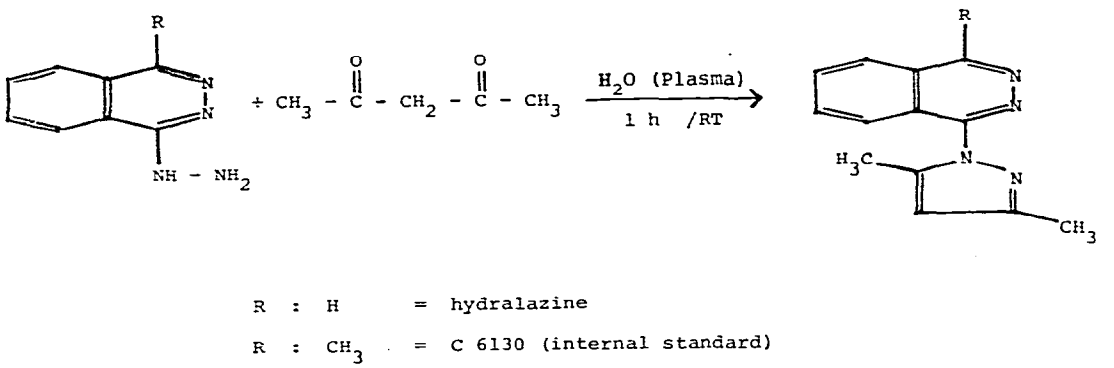

Fig. 1. Formation of the pyrazole derivative of hydralazine and of the internal standard, 4-methylhydralazine. RT = Room temperature.

The stability of hydralazine in native plasma is poor. It is degraded to phthalazine, especially in the presence of oxygen at pH 7.0. In native plasma at a pH of 7.4 a loss of *ca.* 20% of the hydralazine was reported by Reidenberg *et al.*[14] after incubation for 4 h at 37°. At pH 6.4, which is the pH employed after addition of the internal standard solution in the method reported here, hydralazine is stable sufficiently to react completely with 2,4-pentanedione within *ca.* 40–60 min (Fig. 2). Thus a reaction time of 1 h was chosen for the standard procedure. The overall yield (using 1 ml plasma and 300 ng hydralazine) is *ca.* 40% (Fig. 2).

EXPERIMENTAL AND RESULTS

Chemicals and reagents

The following chemicals and reagents were used: Hydralazine hydrochloride

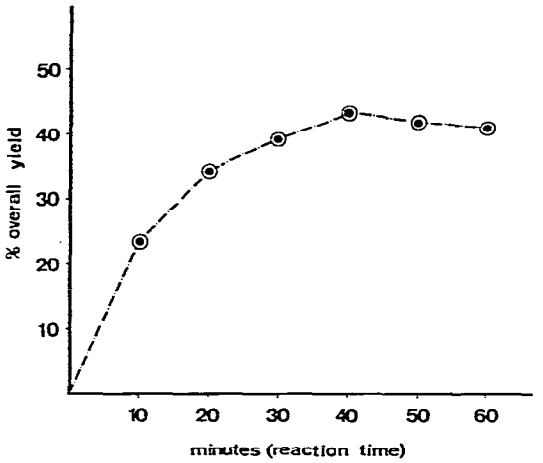

Fig. 2. Reaction kinetics of pyrazole formation in plasma. Percent yield of 1-(3,5-dimethyl-1-pyrazolyl)phthalazine and its dependence on the reaction time.

(Ciba-Geigy, Basle, Switzerland), solutions in 0.1 *N* HCl, freshly prepared; hydralazine pyruvic acid hydrazone (Ciba-Geigy); 4-methylhydralazine hydrochloride (Ciba-Geigy), solutions in 0.1 *N* HCl, freshly prepared; 2,4-pentanedione (EGA-Chemie, Steinheim, G.F.R.; puriss. 99%); *n*-hexane, distilled.

Procedure

A 1-ml volume of plasma, 0.1 ml internal standard solution (294 ng/0.1 ml)* and 0.2 ml 2,4-pentanedione (pH of the reaction mixture, *ca.* 6.4) were thoroughly mixed and shaken for 1 h at 200 rpm on a mechanical (horizontal) rotary shaker. Hexane (2 ml) was then added and the mixture shaken for 10 min at 200 rpm. The organic phase was transferred to a clean vial and evaporated under a stream of nitrogen at 40°. (The vials should not be left in the water-bath longer than necessary.) The residue was redissolved in 0.2 ml hexane and aliquots of 5 μl were injected into the gas chromatograph.

Gas–liquid chromatography

The instrument used was a Pye-104 gas chromatograph equipped with a Perkin-Elmer nitrogen-specific detector. The column was of Pyrex glass (2 m × 2 mm I.D.), packed with 3% OV-17 on Chromosorb W HP (80–100 mesh). Other conditions: carrier gas (helium) flow-rate, 35 ml/min; temperature of column oven, detector and injector, 230°. The retention times under the given conditions were 3.4 min for the hydralazine derivative and 4.6 min for the internal standard derivative.

The structure of the derivative of hydralazine was ascertained by mass spectrometry (Department of Spectroscopy, Ciba-Geigy; spectrum No. 7270).

Specificity for unchanged hydralazine

Blank plasma was spiked with hydralazine in concentrations ranging from 9.5 to 240 ng/ml. The samples were split into two equal parts. To one part were added

* For calibration curves, hydralazine is added in the same manner.

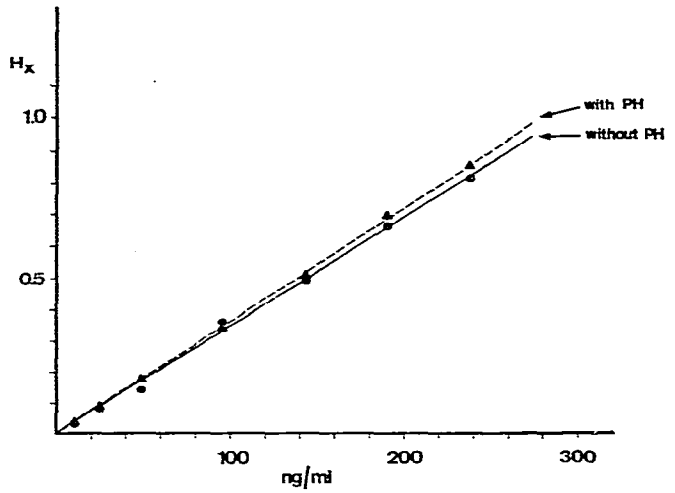

Fig. 3. Specificity test. Calibration curves with and without addition of pyruvic acid hydrazone (PH). Internal standard: 294 ng/ml. Sample volume: 1 ml plasma. ▲, Each sample + 680 ng PH; ●, without PH. H_x = peak height of the derivative of hydralazine/peak height of the derivative of the internal standard.

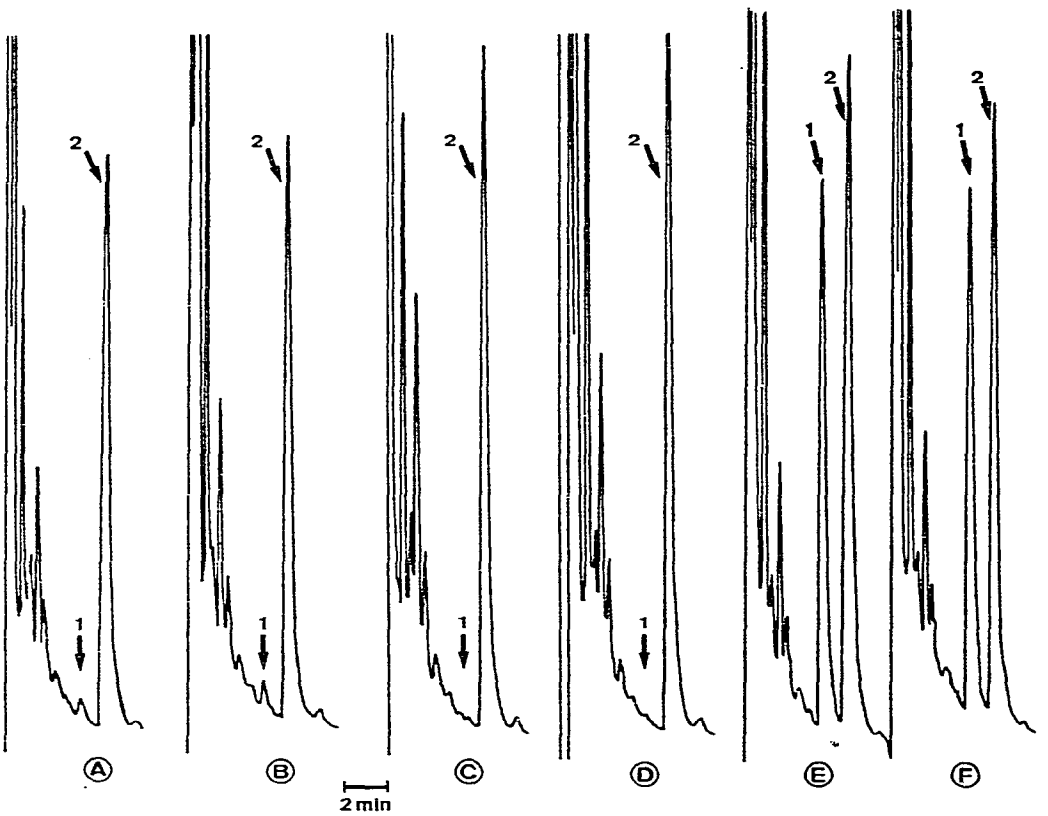

Fig. 4. Chromatograms of extracts of spiked plasma (1 ml) samples. Extracts: A = 9.5 ng hydralazine; B = 9.5 ng hydralazine and 680 ng pyruvic acid hydrazone; C = blank; D = 680 ng pyruvic acid hydrazone. E = 238 ng hydralazine; F = 238 ng hydralazine and 680 ng pyruvic acid hydrazone. A–F also contained 294 ng internal standard. Peaks: 1 = hydralazine derivative; 2 = internal standard derivative. 5-μl aliquots of 0.2 ml were injected.

TABLE I

DETERMINATION OF HYDRALAZINE IN SPIKED PLASMA SAMPLES BY TWO DIFFERENT METHODS

A = Specific method for unchanged hydralazine (reaction with 2,4-pentanedione at pH 6.4); B = method for apparent hydralazine, described by Jack *et al.*[5] (reaction with nitrite at pH 0.2).

Hydralazine in plasma (ng/ml)		
Initial	*Found (method A) ($n = 3$): $\bar{x} \pm s(x)$*	*Found (method B) ($n = 3$): $\bar{x} \pm s(x)$*
236	235 ± 15.56	255.5 ± 3.54
47.2	51 ± 0	52 ± 1.41
147.5	154.5 ± 3.53	148 ± 0
20	19.0 ± 0	20.7 ± 1.2
10	9.7 ± 0.6	9.3 ± 0.8
30	28.0 ± 0	30.0 ± 0

680 ng of the pyruvic acid hydrazone of hydralazine. Both parts were then analyzed as described.

It was found that the hydrazone did not interfere with the analysis of unchanged hydralazine. No difference could be seen between the samples containing hydrazone and the samples containing only unchanged hydralazine (Fig. 3).

The blank plasma sample containing only the 680 ng of the pyruvic acid hydrazone of hydralazine showed no peak in the chromatogram at the position where the hydralazine derivative would appear (Fig. 4).

A comparison between the method reported previously for the determination of apparent hydralazine[5] and the method reported here for unchanged hydralazine has been carried out using spiked plasma samples. Six different pools were prepared. The results (Table I) show good agreement between the two methods. The sensitivity of both procedures is about the same (*ca.* 10 ng/ml).

Application of both GC methods to the analysis of biological samples

Twenty-one male rats (200 g) were treated by gavage with 12 mg/kg doses of hydralazine. Three animals were sacrificed at each of the following times: 0, 0.5, 1,

TABLE II

PLASMA LEVELS OF APPARENT AND UNCHANGED HYDRALAZINE IN RATS TREATED WITH A SINGLE ORAL DOSE OF HYDRALAZINE (12 mg/kg)

0.5–1.0 ml plasma were used for both assays.

Time after administration (h)	*Apparent hydralazine* (ng/ml) ($n = 3$): $\bar{x} \pm s(x)$*	*Unchanged hydralazine** (ng/ml) ($n = 3$): $\bar{x} \pm s(x)$*
0	0	0
0.5	1310 ± 198	98.0 ± 6.9
1	373 ± 32	39.7 ± 0.6
2	264 ± 2.8	29.0 ± 0
3	222 ± 6.4	30.0 ± 4.2
6	82 ± 11	10.0 ± 1.4
24	10 ± 0	0

* GC assay for apparent hydralazine[5].

** GC assay for unchanged hydralazine described in this paper.

2, 3, 6 and 24 h after administration. The plasma samples for each time point were pooled and analysed both for apparent hydralazine according to the method of ref. 5 and for unchanged hydralazine by the present method. The results in Table II demonstrate that between 7.5 and 13.5% of the apparent hydralazine in the plasma of rats can be accounted for as unchanged hydralazine.

The analytical principle described in this paper has been found applicable to the assay of dihydralazine (1,4-dihydrazinophthalazine) in biological fluids. Its detailed description will be given in a subsequent paper.

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