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Note

Determination of apparent dihydralazine in plasma by gas—liquid chromatography and electron-capture detection

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Dihydralazine sulfate, the active ingredient of Nepresol®, is a potent vasodilator. Therapeutic doses are low, normally 25 mg. Measurement of the compound in biological fluids thus requires a method of high sensitivity. It has been shown that after administration of hydralazine, an analogue of dihydralazine, the major fraction of the compound measurable in plasma exists in the form of acid-labile hydrazones such as pyruvic acid hydrazone [1].

Previous methods were either not sensitive enough or have not been optimized to measure apparent dihydralazine [3—10]. The recently published specific method for free dihydralazine [11] is not sensitive enough for the measurement of therapeutic levels of dihydralazine in man.

This paper describes a method to assay apparent dihydralazine in plasma. The method is based on hydrolysis of the acid labile compounds followed by reaction with nitrous acid to form the azidotetrazolophthalazine (II, Fig. 1). This compound when isolated in crystalline form is a violent explosive and thus not thermally stable enough for gas chromatographic (GC) analysis. In a second step, the labile derivative is treated with sodium methylate to form the methoxytetrazolophthalazine (III, Fig. 1). This final derivative is stable and has excellent properties for its GC determination with electron-capture detection. 6-Trifluoromethyl-dihydralazine is used as an internal standard. After derivatisation, this compound forms two isomers. However, the ratio of the two peaks is constant (0.68) and thus any of the two peaks may be used for the quantitation.

Acid-labile hydrazones of dihydralazine in plasma of patients can be hydrolyzed to yield concentrations of apparent dihydralazine far in excess to the free dihydralazine as measurable without preceding hydrolytic treatment of plasma.

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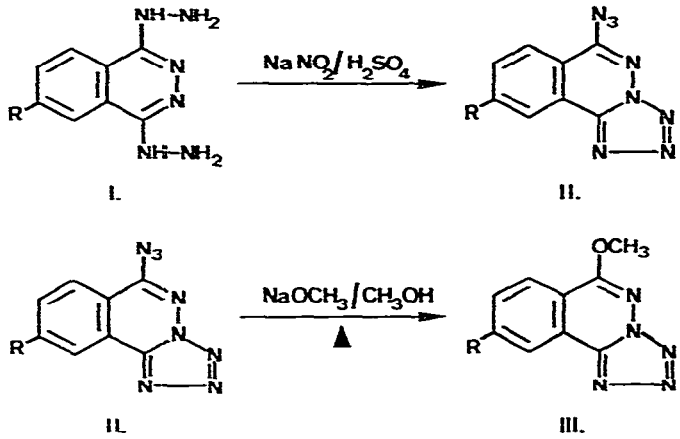

Fig. 1. Structures of dihydralazine (R = H) and 6-trifluoromethyl-dihydralazine (R = CF_3) (I), azidotetrazolophthalazine (II) and methoxytetrazolophthalazine (III).

MATERIALS AND METHODS

Reagents

All standard solutions (dihydralazine sulfate and 6-trifluoromethyl-dihydralazine hydrochloride) were prepared daily in 0.1 *N* hydrochloric acid. Toluene and methanol, laboratory grade, were distilled before use.

Sodium nitrite and sodium methylate were obtained from Fluka (Buchs, Switzerland). Buffer solutions were prepared as follows. pH 13.8 buffer: 0.225 mol potassium hydrogen phthalate and 1.575 mol sodium hydroxide, made up to 1 liter. pH 7.0 buffer: 0.041 mol disodium hydrogen phosphate and 0.028 mol potassium hydrogen phosphate, made up to 1 liter.

Procedure

A 20-ml extraction tube is wetted with 4 ml of 1.5 *N* sulfuric acid. After addition of 1 ml of plasma, 50 µl of internal standard solution (1 ng/µl) are added. The tube is stoppered and agitated in a water bath at 90°C for 25 min (100 rpm). After cooling to room temperature, 0.1 ml of 50% sodium nitrite solution is added, briefly mixed and left at room temperature for 15 min. Then 4 ml of buffer solution pH 13.8 are added to bring the pH to about 4.5. After addition of 5 ml of toluene, the mixture is shaken for 10 min on a horizontal mechanical shaker at 120 rpm. After brief centrifugation, the organic phase is removed and evaporated to dryness under a stream of nitrogen at 40°C. A solution of 8.6 µmol sodium methylate in 1 ml of toluene (with 5% methanol) is added to the dry residue and left at 50°C for 1 h; 3 ml of buffer solution pH 7 are then added and shaken for 10 min at 200 rpm. After brief centrifugation, the organic phase is removed and aliquots of 5 µl are injected into the gas chromatograph.

Gas chromatography

A Pye 204 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. The column was a 1.5 m × 4 mm I.D. Pyrex glass column packed

with 3% OV-225 on Ultrapak Gas-Chrom Q, 230—270 mesh. The carrier gas flow-rate was 50 ml of nitrogen per min. The temperatures were: injector and column oven 250°C, detector 350°C. The retention times for the two derivatives were 6.6 min for the methoxytetrazolophthalazine and 4.0 min for the more abundant isomer of the trifluoromethyl-methoxytetrazolophthalazine. Chromatograms of a blank plasma sample and of a plasma sample spiked with 40 ng of dihydralazine are shown in Fig. 2.

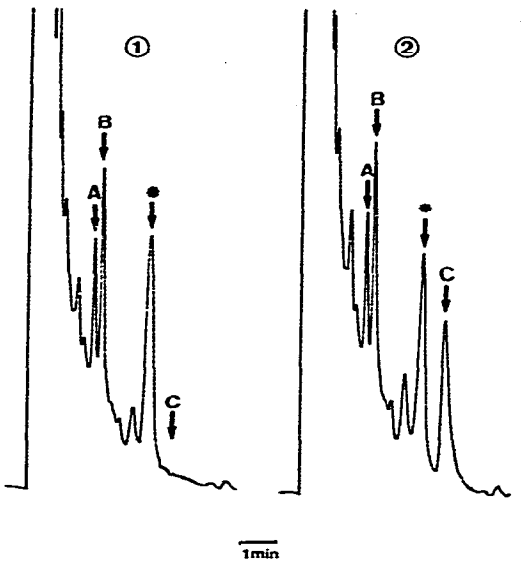

Fig. 2. Typical chromatograms of (1) extract of a blank plasma sample (1 ml) with 50 ng of internal standard, and (2) extract of a plasma sample spiked with 40 ng of dihydralazine sulfate and 50 ng of internal standard. Injected aliquot: 1/200th. The peak marked with an asterisk is an unknown constituent of plasma. (A, B) Isomers of the internal standard derivative (B is used for the quantitation of dihydralazine). (C) Derivative of dihydralazine.

Calibration graph

A calibration graph was prepared as follows. Blank plasma samples were spiked with solutions of dihydralazine in 0.1 *N* hydrochloric acid (0—330 ng/μl). The samples (n = 11) were then processed as described. The peak height of the dihydralazine derivative was divided by the peak height of the internal standard derivative and the ratio (H_x) plotted against initial dihydralazine concentrations.

Calculation of the linear regression resulted in a coefficient of correlation (r) of 0.9999 and a standard error of estimate (S_y) of 0.0086 H_x.

RESULTS AND DISCUSSION

Hydrolysis

Acid strength, time and temperature of incubation were optimized to obtain maximum yield of apparent dihydralazine using plasma pools from rats treated with dihydralazine (12 mg/kg per os). Sulfuric acid up to 6 *N*, incuba-

tion temperatures up to 100°C, and reaction times up to 120 min were tested. It was found that hydrolysis with 1.5 *N* acid at 90°C for 25 min produces optimal recoveries.

Stability

Dihydralazine is rapidly oxidized under alkaline conditions. Only about 20% of unchanged dihydralazine was found after 48 h storage in neutral aqueous solution. However, a solution of dihydralazine in 0.1 *N* hydrochloric acid stored under the same conditions was found to be stable.

Added to plasma, dihydralazine is very unstable, even when stored at —20°C. The addition of hydrochloric acid improves the stability only marginally. Without acid, about 80% of the dihydralazine is lost after 48 h of storage at —20°C, and with hydrochloric acid added (pH 1) about 60% is lost. In contrast to this observation, plasma samples from in vivo studies were kept up to 28 days at —20°C without detectable losses of apparent dihydralazine. The instability of dihydralazine seen in spiked biological samples refers to unchanged dihydralazine only. Dihydralazine proved quite stable in plasma samples obtained from animal studies, when the samples were measured by the GC technique for apparent dihydralazine.

This seeming discrepancy between findings with spiked biological or aqueous samples and with samples obtained from animals after dihydralazine administration, suggests that dihydralazine, like hydralazine [1, 2], forms metabolites which are stable upon storage but regenerate free dihydralazine upon hydrolysis in the analytical procedure.

Derivatisation

The nitrous acid reaction, according to Jack et al. [12], was found to be completed after 15 min at room temperature and the yields in water and plasma were about 80%. In blood the yields drop to about 50—60%.

The formation of the methoxy derivative is complete after 1 h at 50°C. The yields at concentrations up to 500 ng per sample are almost 100%. The final derivative is stable in toluene for several days at room temperature. The structures of the derivatives have been verified by gas chromatography—mass spectrometry (GC—MS).

The internal standard forms two isomers (6-trifluoro-4-methoxy- and 6-trifluoro-1-methoxy-tetrazolophthalazine) upon derivatization. Both isomers have been verified by GC—MS; however, on the basis of molecular ion and fragmentation data it is not possible to differentiate between the two isomers. The ratio between the two derivatives is very stable; it was found to be 0.68 ± 0.05 (mean ± S.D.) calculated from a series of 40 chromatograms. Thus, for convenience, the larger of the two peaks was chosen for the evaluation of the chromatograms.

Recovery, precision and limit of quantitation

Recovery of dihydralazine and precision were evaluated by analysing spiked samples. Fifteen samples were prepared with dihydralazine concentrations between 10 and 130 ng/sample. The differences between the found and the initial concentrations were between —0.6% and +12.2%.

Calculation of the linear regression between given and found concentrations resulted in a coefficient of correlation (r) of 0.9997 and a standard error of estimate (S_y) of 1.1927 ng.

The limit of quantitation is about 5 ng/ml plasma.

Application

One healthy, male volunteer received 12.5 mg (½ tablet) of Nepresol® in the morning 2 h before breakfast. Blood samples were collected immediately before and at specified times after administration of the oral dose. The blood samples were immediately centrifuged, the plasma removed and analysed as described above.

The peak plasma level was 87 ng/ml, attained 1 h after administration (Fig. 3). The plasma levels found are considerably higher than those found in an earlier study, where the acid-labile components were not completely hydrolysed [10]. However, the apparent biological half-life values are comparable (2—3 h).

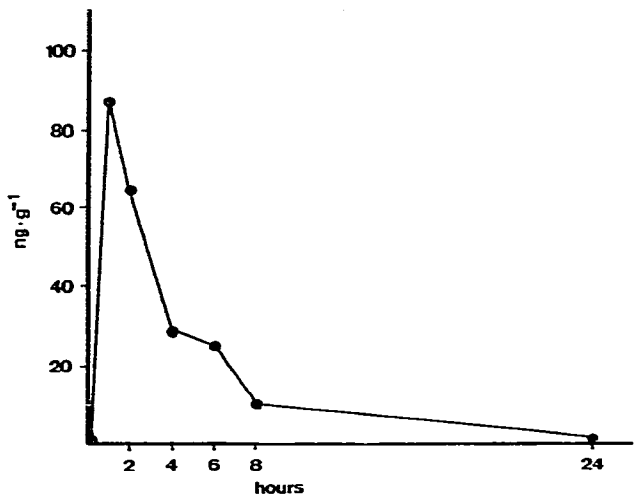

Fig. 3. Plasma levels of apparent dihydralazine after a single, oral dose of 12.5 mg of Nepresol in a healthy volunteer.

No measurable concentrations of free dihydralazine were found after analysis of the samples by the GC method with nitrogen-specific detection [11] (detection limit: ca. 20 ng/ml).

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