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Liquid chromatographic determination of hydralazine in human plasma with 2-hydroxy-1-naphthaldehyde pre-column derivatization*

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Abstract: A selective and sensitive high-performance liquid chromatographic method is described for determination of hydralazine and its metabolites in human plasma. The method involves pre-column derivatization with 2-hydroxy-1-naphthaldehyde at pH 1.2. The reaction product and Methyl Red used as internal standard are extracted into dichloromethane and chromatographed in the reversed-phase mode on an ODS-2 column using acetonitrile–aqueous triethylamine phosphate buffer (80:20, v/v) at pH 3 as eluent.

The plasma calibration curve of hydralazine is linear in the concentration range 10–500 ng ml^{-1}. The detection limit is 1 ng ml^{-1} and the relative standard deviation is <2.4. *In vivo* pharmacokinetics of hydralazine in two volunteers after oral administration of 50 mg of the drug is studied using the proposed LC method.

Keywords: *Hydralazine determination; 2-hydroxy-1-naphthaldehyde derivatization; reversed-phase HPLC; pharmacokinetics.*

Introduction

Hydralazine is one of the most commonly used drugs in the treatment of essential hypertension because of its peripheral vasodilator effect [1]. The determination of hydralazine in biological samples of humans who have ingested the drug is an essential requirement to the understanding of the clinical effects of hydralazine and the determination of its pharmacokinetics parameters.

Several methods have been described for the determination of hydralazine in the underivatized form involving high-performance liquid chromatography with ultraviolet detection that are suitable for the assay of pharmaceutical dosage forms [2, 3], for monitoring the metabolism of hydralazine by rat liver microsomes [4] and the assay of hydralazine and its metabolites in serum, plasma and urine [5]. In addition, a HPLC method with electrochemical detection has been proposed for direct determination of hydralazine and metabolites [6].

Other specific assays have been developed using derivatizing agents for hydralazine in order to increase the selectivity and sensitivity of the determination. Thus, several analytical procedures have been reported which involve pre-column derivatization of hydralazine with 2,4-pentanedione [7] followed by GLC with nitrogen-specific detection. In other cases, HPLC is used after derivatization with either sodium nitrite using fluorimetric detection [8, 9] or with *p*-anisaldehyde and *p*-nitrobenzaldehyde using spectrophotometric detection in the visible region [10–12].

This report describes a specific and sensitive assay for hydralazine and its metabolites in human plasma by derivatization with 2-hydroxy-1-naphthaldehyde followed by LC with Methyl Red as internal standard.

Experimental

Apparatus

A Shimadzu Model LC-6A liquid chromatograph equipped with a variable-wavelength UV–vis detector (Shimadzu Model SPD-6AV) and a computing integrator (Shimadzu Model C-R6A) was used. Samples were injected through a Rheodyne valve injector Model 7125 fitted with a 20-μl loop. The analytical column (250 × 4 mm i.d.) packed with Spherisorb

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ODS-2, particle size 3 μm (Tracer Analítica S.A. Barcelona, Spain) was used. The aqueous portion of the mobile phase contained 0.75% phosphoric acid (v/v) and 0.5% triethylamine (v/v) in distilled water. The mobile phase was acetonitrile–buffer solution (80:20, v/v) pH 3, pumped at a flow rate of 0.7 ml min^{-1}. Experiments were carried out at room temperature with a detection wavelength of 406 nm.

Millipore XX047 filtration equipment with a Millipore HVLP 04700 filter was used.

Reagents

All reagents were analytical grade. Acetonitrile of HPLC grade purity was used. Aqueous solutions were prepared using in glass distilled water.

The internal standard, Methyl Red (500 μg ml^{-1}) was prepared in methanol. A stock solution of hydralazine hydrochloride (500 μg ml^{-1} of free hydralazine base) from Ciba-Geigy, stored at 4°C was used without further purification. A working standard solution of 1 μg ml^{-1} was prepared daily.

2-Hydroxy-1-naphthaldehyde (HNA) (Aldrich Chemical) 0.01 M was prepared in ethanol.

The reagents used as eluents in liquid chromatography were filtered immediately before use.

Blood collection and storage

Blood samples (5 ml) were collected in heparinized tubes and were immediately centrifuged at 1500 rpm for 5 min to separate plasma. Two 1-ml aliquots of plasma were put into separate glass tubes, and stored at −18°C.

Procedure

To 1 ml of plasma in a borosilicate glass-stoppered tube were added 0.02 M EDTA-Na_2 (1 ml), 0.5 M HCl (1 ml) and 0.01 M HNA (1 ml). The tube contents were mixed for 15 s on a vortex mixer and kept in a water bath at 25°C for 90 min. Internal standard (50 μl) and dichloromethane (7 ml) were added. The tube was then shaken for 5 min on a vortex mixer and centrifuged at 4500 rpm for 15 min. The organic phase was transferred into a 10-ml borosilicate glass tube, evaporated to dryness at 50°C under a gentle stream of nitrogen and subsequently redissolved in 100 μl of acetonitrile, 20 μl of which were injected into the chromatograph.

Results and Discussion

The method involves reaction of plasma samples with HNA to yield a water insoluble yellow product, 1-[(2-hydroxy-1-naphthyl) methylenhydrazino]phthalazine (HNMP). This product is slightly unstable in sunlight. Use of borosilicate glass tubes is found to give better precision.

Figure 1 shows the chromatograms of (A) untreated plasma, (B) 1 ml of a plasma sample spiked with 25 ng ml^{-1} of hydralazine and (C) 1 ml plasma from a 70-kg volunteer 1.5 h after a 50-mg single oral dose of hydralazine. Good baseline separation was achieved between HNPH, the internal standard Methyl Red and the excess reagent. The use of a buffer solution of pH ≈3 in the mobile phase is essential for the efficient and quantitative elution of HNPH, which possesses a phenolic hydroxyl group.

4-Methylhydralazine, which is the internal standard usually employed for hydralazine determination in biological samples [7–11], is not commonly available. For this reason, Methyl Red a common laboratory reagent was selected as internal standard.

The plasma calibration curve of hydralazine is linear in the concentration range 10–500 ng ml^{-1} with a correlation coefficient of 0.9997. The mean relative standard deviation (RSD) for replicate analysis of spiked plasma samples is <2.4%. The limit of detection (determined at peak height twice baseline noise) was 1 ng ml^{-1} using 1 ml of plasma.

The recovery of hydralazine from plasma was determined from samples of plasma spiked with 40, 80 and 120 ng ml^{-1}. The samples were treated as specified in the experimental procedure and the chromatogram peak area ratios of hydralazine and Methyl Red were compared with the peak area ratios obtained from aqueous solutions of same concentrations. The extraction recovery of hydralazine from plasma was found to be 50–55% compared with that from water. For this reason, it is necessary to generate the calibration curve using untreated plasma samples. No interferences were observed with the normal constituents of plasma and other hydrazine drugs at ng ml^{-1} levels, such as iproniazid, nialazide, phenelzine and isoniazid reaction products of which with HNA are colourless. Dihydralazine, also used as an antihypertensive drug gives a yellow hydrazone that produces a peak at 14.2 min under these

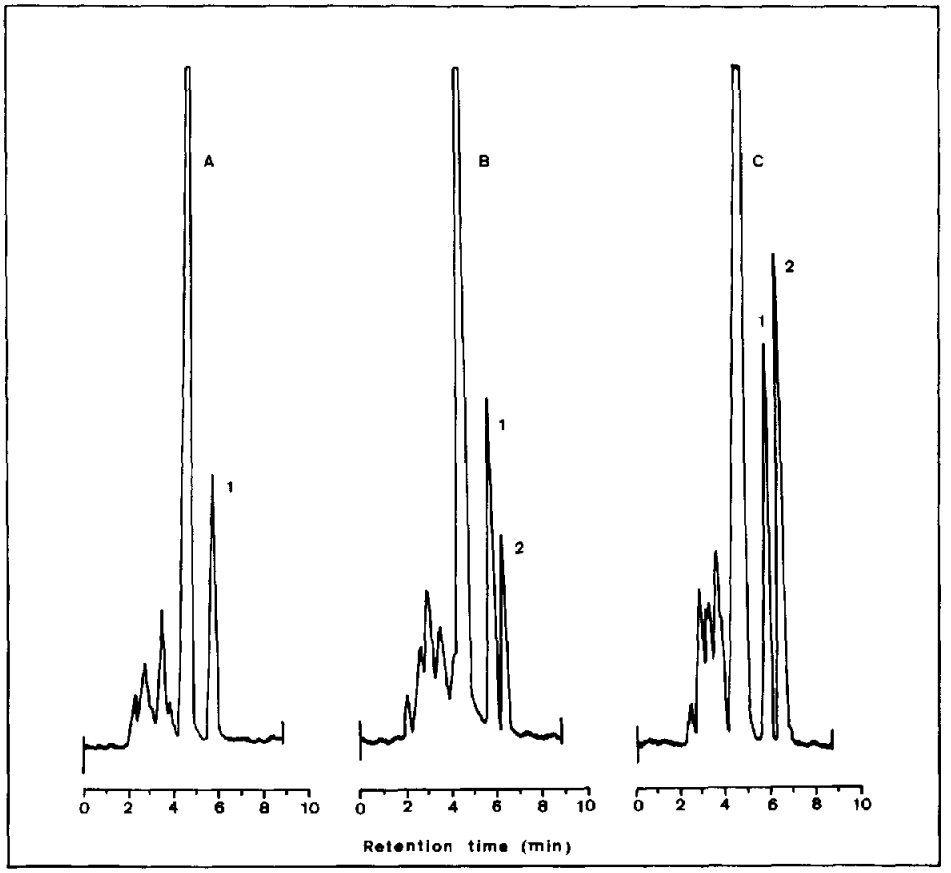

Figure 1
Chromatogram of (A) untreated plasma (1, internal standard), (B) plasma spiked with 25 ng ml^{-1} of hydralazine (1, internal standard; 2, reaction product), (C) plasma sample from a volunteer after a single oral dose of 50 mg of hydralazine. Experimental conditions as described in text.

chromatographic conditions, which is well resolved from HNMP (6.4 min).

The present procedure determines the apparent hydralazine levels, that is, hydralazine plus its metabolites. These metabolites are acid labile hydrazones which are converted back to hydralazine by acidic conditions.

Figure 2 shows human concentrations versus time after administration of a single 50-mg oral dose of hydralazine to two volunteers of 70 (A) and 55 kg (B) weight, respectively. The maximum plasma concentration (C_{max}) was of 246 and 215 ppb, respectively, and the maximum time corresponding to C_{max} was 0.66 to 1.50 h. The maximum concentrations reached in plasma were in agreement with those reported by Haegele *et al.* [5] and Reece *et al.* [8] but higher than that reported by Rouan *et al.* [9] and Semple *et al.* [11]. The

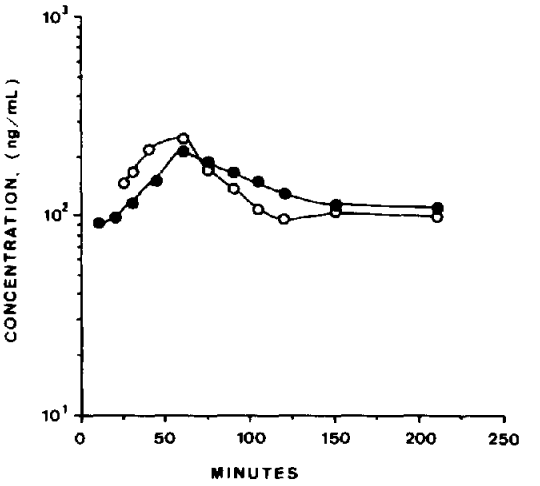

Figure 2
Log-linear plot of plasma concentration versus time after a single oral dose of 50 mg of hydralazine to two volunteers (○) 70 kg (●) 55 kg.

Table 1
Pharmacokinetics parameters from two volunteers of 70 kg (A) and 55 kg (B) weights treated with a single oral dose of hydralazine (50 mg)

Volunteer	t_{max} (h)	K_a (h^{-1})	$t_{1/2}$ (h)	K_{el} (h^{-1})	$t_{1/2}$ (h)	C_{max} (ng ml^{-1})
A	0.83	0.196	3.536	0.878	0.789	246
B	1.00	1.608	0.431	0.480	1.442	215

terminal half-life measured in this study was 0.79 h for volunteer A and 1.66 h for volunteer B. The results obtained in this study are illustrated in Table 1.

This method provides a sensitive, selective and simple procedure for the determination of apparent hydralazine concentrations in human plasma.

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