*Journal of Chromatography*, 176 (1979) 480–484


CHROM. 11,946

## Note

# Liquid chromatographic analysis of hydralazine and metabolites in plasma

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(Received April 23rd, 1979)

Hydralazine is an economical effective drug which may be used to treat hypertension and chronic, resistant congestive heart failure. While this drug has been in use for quite some time, the complete metabolism and the pharmacokinetics of hydralazine remain undefined. The complexity of the problem may be in part due to the fact that hydralazine is chemically very reactive; it readily forms hydrazones with biogenic aldehydes and ketones. High-pressure liquid chromatographic (HPLC) studies of the reaction of hydralazine with several aldehydes and ketones at physiological conditions have been effected in this laboratory[1,2]. It was found that the reactions of hydralazine with formaldehyde and acetaldehyde are of special interest, because these reactions lead to the formation of *s*-triazolo[3,4*a*]phthalazine (I) and 3-methyl-*s*-triazolo[3,4*a*]phthalazine (II), respectively, with the hydrazones as intermediates. Both I and II have been reported to be the metabolites of hydralazine[3,4]. In fact, II is the major metabolite accounting for 80% of the total drug in urinary excretion, whereas I is present only in trace amounts in the urine[5].

In order to obtain reliable pharmacokinetic data, the assay procedure used must be highly sensitive and selective. Most of the early studies on hydralazine were based on a spectrophotometric assay[6] which, unfortunately, does not allow one to distinguish between unchanged drug and metabolites[7]. Improved methods have since been published using gas–liquid chromatographic techniques[8,9]. While these methods have been shown to be successful in the analysis of hydralazine, the present study introduces an alternative assay procedure using HPLC. This HPLC method is based on the conversion of hydralazine into compound I with formaldehyde under acid condition. Although I has been shown to be a metabolite of the drug, its concentration in plasma or serum samples is generally non-detectable. The advantages of this method will be discussed.

## EXPERIMENTAL

### *Materials*

Hydralazine (kindly supplied by Ciba-Geigy, Summit, N.J., U.S.A.), formaldehyde and 8-chlorotheophylline (both from Aldrich, Milwaukee, Wisc., U.S.A.) were used as obtained. High-purity samples of I and II were prepared by preivous

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literature procedures[10,11] and used as standards. Human plasma was obtained from a local blood bank. All solvents used were HPLC grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.).

*Equipment*

A HPLC system, including a Model 6000 A pump and a U6K injector (Waters Assoc., Milford, Mass., U.S.A.) equipped with Model SF-770 UV and Model SF-970 fluorescence detectors (both from Schoeffel, Westwood, N.J., U.S.A.), was used. The output of the detectors were displaced on a recorder (Omniscribe, Houston Instruments, Austin, Texas, U.S.A.) having a full-scale range of 10 mV. The HPLC analysis was made with a reversed-phase μBondapak $C_{18}$ column (30 cm × 4 mm I.D.) (Waters Assoc.).

*Chromatographic conditions*

The mobile phase was acetonitrile–sodium acetate buffer (0.01 *M*, pH 4) (13:87). The column temperature was ambient. Chromatographic analysis was made with a flow-rate of 1.0 ml/min. The sample injection size was 8 μl. The column eluate was monitored by UV detection at 240 nm and by fluorescence detection with excitation at 240 nm and emission above 389 nm. The UV detector was operated at 0.01 a.u.f.s., the fluorescence detector was operated at 0.2, 0.5 or 1 μA.

*Sample preparation*

Stock solutions of 1 mg/ml of hydralazine and compounds I and II were prepared in methanol. The solutions of authentic I and II were used to identify plasma hydralazine and metabolite in the chromatographic analysis. Synthetic plasma samples were prepared by transferring desired amounts of hydralazine stock into 1 ml plasma with a microsyringe. To convert hydralazine to I, 10 μl of formaldehyde and 10 μl of glacial acetic acid were added to 1 ml plasma containing hydralazine. The resulting plasma sample has a pH of about 5 and contains excess amount of formaldehyde. The sample was then shaken and allowed to stand for 20 min for the completion of the reaction. For blood sample analysis, the plasma obtained by rapid centrifugation of the blood sample were used with and without the formaldehyde treatment. Prior to the analysis; 10 μg/ml of 8-chlorotheophylline was added to all the samples and used as an internal standard for the UV detection.

## RESULTS AND DISCUSSION

The problem associated with the analysis of hydralazine is that the drug is notoriously unstable in aqueous solutions. Studies[5] have shown that hydralazine undergoes rapid degradation in aqueous solution depending on the pH conditions and buffer systems. In solutions containing aldehydes or ketones, such as in biological fluids, hydralazine forms hydrazones in an equilibrium process. These phenomena suggest that direct measurement of hydralazine will only give results with poor accuracy. A reasonable approach in the developement of a hydralazine assay would be to derivatize the drug into a stable and easily measured derivative. An example of this approach has been shown by Jack *et al.*[8], in which hydralazine was converted to a tetrazolo phthalazine derivative with sodium nitrite. The reaction of hydralazine

with formaldehyde, which occurs rapidly in aqueous solutions under acid condition, provides an excellent means for the determination of hydralazine in plasma samples. Hydralazine is non-fluorescent and only shows moderate UV absorption. Compound I, the derivative of hydralazine with formaldehyde, however, shows marked enhancement of UV absorption as well as a strong fluorescence at 425 nm, when excited at 240 nm.

The chromatographic analysis of a plasma sample containing 200 ng/ml hydralazine is shown in Fig. 1, where the top chromatograms were obtained with a fluorescence detector, and the bottom chromatograms were obtained with a UV detector. Fig. 1A shows the untreated sample, where peak a represents 8-chlorotheophylline which was used as an internal standard for the UV detection. The added hydralazine was not detectable under the chromatographic conditions. Fig. 1B represents the formaldehyde-treated sample, where a new peak b (or b*) appears with a retention time of 13 min. This new peak was found to be identical in retention time and peak response with a sample containing the authentic I. Fig. 1C represents the same sample of 1B, but the fluorescence detection is monitored at a higher sensitivity setting. Using the fluorescence detector, this method allows one to measure hydralazine levels as low as 3 ng/ml. The minimum detection using the UV detector was found to be about 0.1 $\mu$g/ml. The standard curve for the hydral-

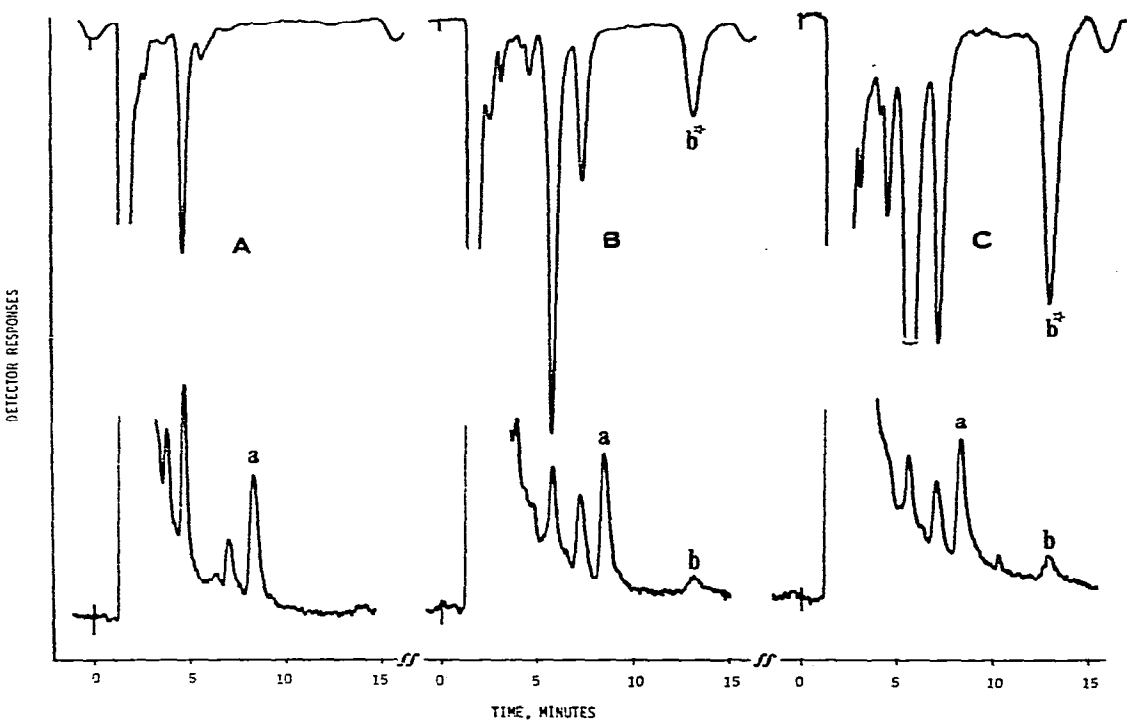


Fig. 1. The UV (bottom) and fluorescence (top) chromatograms of a synthetic plasma sample containing 200 ng/ml hydralazine and 8-chlorotheophylline. (A), Untreated sample, fluorescence range is 0.5 $\mu$A; (B), formaldehyde-treated sample, fluorescence range is 0.2 $\mu$A; (C), as (B), except for a higher sensitivity setting for the fluorescence detection. The UV detection is set at 0.01 a.u.f.s. Peak a represents 8-chlorotheophylline (internal standard), peak b (or b*) represents compound I.

azine analysis of plasma samples using a fluorescence detector is shown in Fig. 2. It can be seen that this method is applicable to the entire range of hydralazine plasma levels. A standard curve for the determination of II, the major metabolite of hydralazine, was also obtained with the same sensitivity. Under the same chromatographic condition, compound II exhibits a retention time of 24 min.

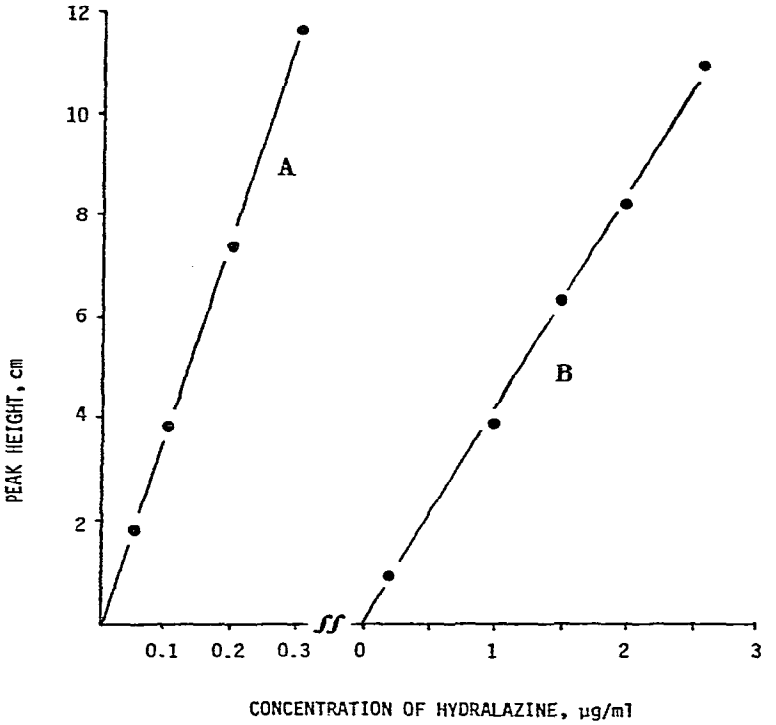


Fig. 2. A calibration curve for hydralazine in synthetic plasma samples with the fluorescence detection. (A), Fluorescence range of 0.2 $\mu$A; (B), fluorescence range of 1.0 $\mu$A.

Fig. 3 shows the chromatographic analysis of a plasma sample obtained from a patient. The formaldehyde-treated sample is shown by the dashlined chromatograms a patient. The formaldehyde-treated sample is shown by the dashlined chromatograms. It can be seen that the presence of II is indicated by peak c (or c*). When the sample is treated with formaldehyde, the hydralazine level can be easily measured by peak b (or b*).

The method described in this study allows one to use directly plasma samples without protein precipitation or extractions. If one prefers to extract the components after the formation of I with organic solvents, the water soluble constituents in the plasma shown in Fig. 1 and 3 may be removed. In this case, it is more advantageous to use a higher concentration of acetonitrile (up to 20%) in the mobile phase, because at a higher acetonitrile concentration, the peaks of I and II appear at reduced retention times with higher resolution.

Although compound I has been shown to be a minor metabolite of hydralazine its concentration in plasma samples is usually low and non-detectable. There-

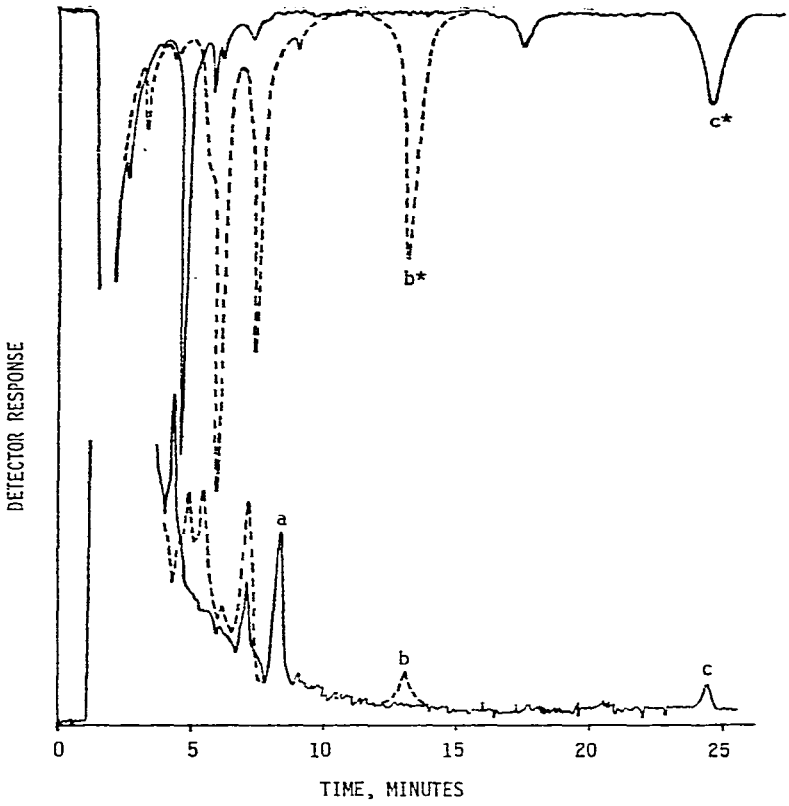


Fig. 3. The UV (bottom) and fluorescence (top) chromatograms of a human plasma sample. ——, Untreated; – – – –, treated with formaldehyde. The fluorescence range was set at 0.5 $\mu$A. The UV detection was operated with 0.01 a.u.f.s. Peak a represents 8-chlorotheophylline, b (or b*) represents compound I, c (or c*) represents compound II.

fore, no interference is expected. At high doses of hydralaine, compound I may be present in the plasma in measurable quantities. In this case, the concentration of I may be first quantitated prior to the treatment with formaldehyde.

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