Journal of Chromatography, 385 (1987) 261–266
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1040

DETERMINATION OF HYDRALAZINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Hydralazine is used as an antihypertensive vasodilator drug. A specific and sensitive method for extraction and analysis of hydralazine by high-performance liquid chromatography (HPLC) with electrochemical detection was developed. Hydralazine and 4-methylhydralazine (internal standard) in plasma were derivatized at room temperature with salicylaldehyde. The derivatives were extracted in basic medium with a mixture of heptane, methylene chloride and isopentyl alcohol. A very good separation of hydralazine and 4-methylhydralazine from matrix material was achieved on a Supelcosil LC-18-DB (5 μm) reversed-phase column kept at 28°C with a mobile phase of 66% methanol in 0.055 *M* citric acid/0.02 *M* dibasic sodium phosphate (pH 2.5). The hydralazine level was measured electrochemically by a screen oxidation mode. This method offers significant advantages in sensitivity, specificity and accuracy. Sample analysis by HPLC required less than 8 min. Application of the method to monitor plasma levels of hydralazine from a patient receiving the drug for the treatment of severe pregnancy-induced hypertension is discussed.

INTRODUCTION

Hydralazine (Apresoline, 1-hydrazinophalazine) has been used for years as a potent vasodilator in the treatment of hypertension. Despite its long history of use, it is often necessary to study the biological disposition and pharmacokinetic characteristics of the drug to ensure its efficacy in treating hypertensive patients. A sensitive and specific analytical method for the accurate measurement of hydralazine concentrations in biological fluids is therefore of paramount importance.

Several analytical methods for measuring hydralazine concentration have been proposed. These include spectrophotometric techniques[1–4], inverse isotope dilution analysis[3,5], gas–liquid chromatography (GLC) with electron capture detection[4,6], GLC with nitrogen-specific detection[7–11], GLC with mass spectrometry[12,13], high-performance liquid chromatography (HPLC) with fluorometric detection[14,15], and

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HPLC with UV detection[14,16,17]. These methods for the determination of hydralazine vary in complexity, sensitivity, and specificity.

Since hydralazine is very unstable in plasma or blood[15,18,19], a rapid sample derivatization is needed. An acid-labile hydrazone, identified as the pyruvic acid hydrazone of hydralazine, has been reported as a major metabolite in human plasma[7,15,17,18]. This metabolite is rapidly converted back to hydralazine under acidic conditions[7,18], thereby interfering with the analysis of unchanged hydralazine.

Hydralazine forms hydrazone derivatives with aldehydes, ketones, and some keto carboxylic acids in biological fluids[2,12,14,17,20]. The hydrazones of hydralazine with acetaldehyde, acetone, and α-ketoglutaric acid have already been identified as metabolites of hydralazine[2,12,20]. Derivatization of hydralazine in human plasma with *p*-anisaldehyde (*p*-methoxybenzaldehyde) to form *p*-anisaldehyde hydrazone has been described[16,17]. We have recently investigated reversed-phase HPLC with electrochemical detection (ED) as a method of high specificity for the determination of hydralazine in human plasma, in which salicylaldehyde was used as a derivatizing agent. 4-Methylhydralazine was used as an internal standard. Hydralazine and the internal standard were derivatized at room temperature to form the salicylaldehyde hydrazones of hydralazine and the internal standard, respectively. It is the purpose of this paper to describe a sensitive and selective analytical procedure for the measurement of hydralazine by HPLC–ED and to illustrate that this method can be used for the clinical quantitation of hydralazine in patients with severe pregnancy-induced hypertension.

EXPERIMENTAL

Chemicals

All reagents were of highest purity available. Hydralazine hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.), 4-methylhydralazine (internal standard) and hydralazine pyruvic acid hydrazone were generously supplied by Ciba-Geigy (Basle, Switzerland). Standard solutions of hydralazine hydrochloride and 4-methylhydralazine were freshly prepared in 0.1 *M* hydrochloric acid and stored at 4°C. A solution of pyruvic acid hydrazone was prepared in 0.01 *M* sodium hydroxide. Salicylaldehyde and ascorbic acid (Sigma) were used in preparing the derivatization reagent. Heptane, dichloromethane (ACS-certified and HPLC grade; Fisher Scientific, Fair Lawn, NJ, U.S.A.), isopentyl-alcohol (Aldrich, Milwaukee, WI, U.S.A.) and sodium hydroxide (Sigma) were used in the extraction of the sample. Citric acid, dibasic sodium phosphate (Sigma) and methanol (ACS-certified and HPLC grade, Fisher Scientific) were used to prepare the mobile phase. Dichlorodimethylsilane (Sigma) in toluene was employed for the silanization of glass tubes. Distilled, deionized water was used throughout the protocol.

Sample collection, derivatization, and extraction

Glass tubes used in the evaporation step were silanized with 6% dichlorodimethylsilane in toluene, rinsed with toluene, then methanol, and dried. Screw-cap tops with PTFE liners were used in the preparation of the samples.

Whole blood was rapidly collected in tubes with EDTA. The collected blood was immediately centrifuged at 2000 *g* for 1 min, and 1 ml of plasma was quickly

transferred to a 12-ml round-bottom culture tube, containing 100 μl 0.2 *M* ascorbic acid. 4-Methylhydralazine (50 ng) and 10 μl salicylaldehyde were added immediately, and the tube was sealed with a PTFE-faced screw-cap and immediately mixed on a vortex mixer for 10 s. The sample was allowed to stand for 8 min for the complete formation of the salicylaldehyde hydrazone of hydralazine and 4-methylhydralazine. After derivatization, 100 μl 1 *M* sodium hydroxide and 4 ml of an organic solvent mixture consisting of 95.3% heptane, 4% dichloromethane, and 0.7% isopentyl alcohol were added. The sample was shaken vigorously for 5 min and then centrifuged at 2000 *g* for 2 min. The organic phase was transferred to a clean test-tube. The extraction was repeated, and the combined organic phases were evaporated under nitrogen at 30°C. The sample residue was dissolved in 200 μl methanol and stored at 4°C until HPLC analysis. The level of hydralazine was determined by injecting 10 μl of the derivatized sample into the HPLC system.

A standard curve was prepared by spiking control plasma with hydralazine and 4-methylhydralazine, derivatizing and extracting the sample as described above.

High-performance liquid chromatography

The levels of the hydralazine in the methanol suspension were determined by means of HPLC–ED. A Model 6000 A solvent delivery system, a Model 730 data module (Waters Assoc., Milford, MA, U.S.A.), and Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) were used with the HPLC system. The analytical column, 150 × 4.6 mm I.D., was packed with Supelcosil LC-18-DB 5-μm particle size (Supelco, Bellefonte, PA, U.S.A.). The guard column was packed with RP-C_{18} (Alltech Assoc., Deerfield, IL, U.S.A.). The electrochemical detector used was a Model 5100 A Coulochem electrochemical detector control module with Model 5021 conditioning cell and Model 5011 high-sensitivity analytical cell (ESA, Bedford, MA, U.S.A.). The analytical column was maintained at 28°C with a temperature control module (Waters). Chromatography was performed at a flow-rate of 1.5 ml/min with a mobile phase of 66% methanol in 0.055 *M* citric acid/0.02 *M* dibasic sodium phosphate (pH 2.5). The derivatives were detected by the Coulochem electrochemical detector with the following screen oxidation parameters: conditioning cell potential at +0.20 V, detector 1 (coulometric electrode) at +0.25 V, and detector 2 (amperometric electrode) at +0.60 V.

RESULTS AND DISCUSSION

We found that isopentyl alcohol, used in the organic solvent mixture, minimized the formation of an emulsion during extraction of plasma samples. An addition of 0.7% isopentyl alcohol was chosen, because an amount higher than 1% alcohol tended to extract more endogenous impurities from the plasma, which would interfere with the accurate measurement of hydralazine. The extraction efficiency was 94%. The coefficient of variation of the peak height ratios of hydralazine and internal standard was 2.2%, based on the average of twelve independent analyses. For the reproducibility of the retention time of hydralazine a coefficient of variation of 0.6% was obtained. The detection limit of the HPLC analysis was about 200 pg/ml plasma by screen oxidation in the electrochemical detector.

Fig. 1 shows the current–voltage curves for hydralazine salicylaldehyde hy-

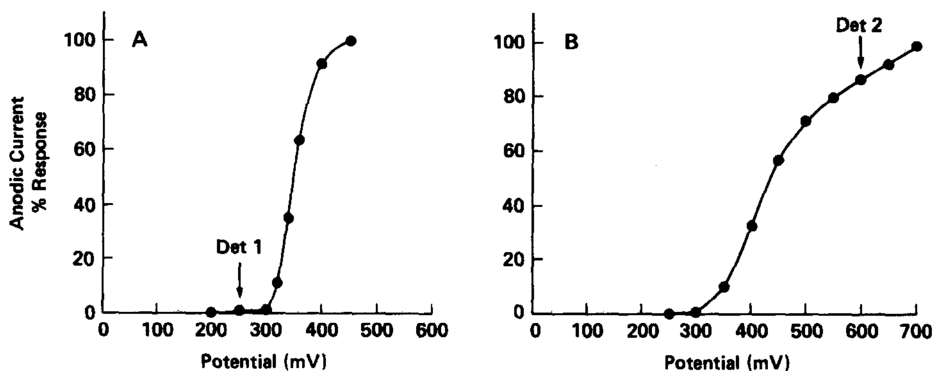

Fig. 1. Current–voltasge curves for hydralazine salicylaldehyde hydrazone. (A) Coulometric response for detector 1 (operating potential selected at +250 mV). (B) Amperometric response for detector 2 (optimum operating potential selected at +600 mV).

drazone. The voltammetric characteristics of the hydralazine derivative were generated by making repetitive injections of a constant amount of the same analyte but varying the electrochemical potential of the detector. Detector 1 was operated coulometrically at an applied potential of +0.25 V and used as a screen detector to oxidize other electrochemically active components present in the plasma up to a potential of +0.25 V (Fig. 1A). Following the elimination of the impurities by the first electrode, the second detector was used to detect the analyte for quantitation. The background current increased considerably above +0.65 V at detector 2 and became a significant fraction of the total current. Therefore, a potential of +0.60 V was selected for detector 2 (Fig. 1B) to measure the unchanged hydralazine derivative amperometrically because at this potential a more selective and enhanced detection was achieved.

Pyruvic acid hydrazone, one of the major metabolites of hydralazine, has been documented to regenerate hydralazine under low pH conditions[7,18]. Since this hydrazone has been a major source of interference in the analysis of unmetabolized hydralazine in human blood[7,15,17,18], we also investigated the specificity of our method. Two control experiments were performed. Control plasma samples (free of the drug and its metabolites), spiked with hydralazine in concentrations ranging from 25 to 500 ng/ml, were analyzed with pyruvic acid hydrazone added in the same concentration range and without pyruvic acid hydrazone. The peak-height ratios of hydralazine and internal standard were then measured. Two almost identical curves were obtained with correlation coefficients of 0.994 for hydralazine with pyruvic acid hydrazone present and 0.998 for hydralazine without the pyruvic acid hydrazone, proving that the reliability of the measurement of free hydralazine under the analytical conditions described was not affected by the possible presence of pyruvic acid hydrazone.

Fig. 2 presents the HPLC separation of hydralazine detected electrochemically with detector 1 operated at +0.25 V and detector 2 at +0.60 V. All analyses were performed under the conditions stated above with isocratic elution. Neither hydralazine nor the internal standard were detectable without derivatization (Fig. 2A). Resolution of hydralazine and the internal standard could be effected in 8 min, as

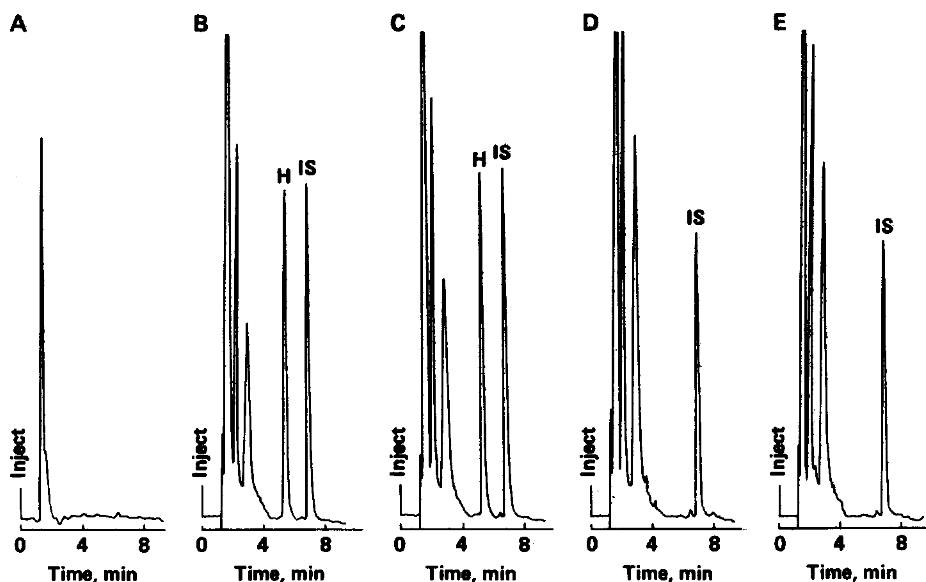

Fig. 2. Chromatograms of extracts of control plasma samples (1-ml samples) spiked with 100 ng/ml internal standard (IS): (A) 100 ng hydralazine without derivatizing agent salicylaldehyde, (B) 100 ng hydralazine and IS, (C) 100 ng hydralazine and IS with 100 ng pyruvic acid hydrazone, (D) IS, (E) IS with 100 ng pyruvic acid hydrazone. Conditions: injection volume: 10 μl; column, Supelcosil LC-18-DB, 5-μm, 150 × 4.6 mm; mobile phase, 66% methanol in 0.055 M citric acid/0.02 M dibasic sodium phosphate (pH 2.5); flow-rate, 1.5 ml/min; electrochemical detectors, Model 5100 A Coulochem; detector 1, +0.25 V; detector 2, +0.60 V; conditioning cell, +0.20 V; column temperature, 28°C.

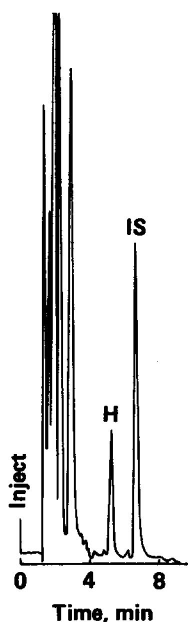

Fig. 3. Chromatogram of hydralazine in plasma from a patient with severe pregnancy-induced hypertension receiving hydralazine. For conditions, see Fig. 2.

shown in Fig. 2B and C. Because of the efficient sample clean-up, combined with the high specificity of the electrochemical detector, no endogenous extractives eluted with the retention times of hydralazine and of the internal standard (Fig. 2D and E) interfered. It is apparent that pyruvic acid hydrazone does not interfere with the quantitation of free hydralazine in plasma (Fig. 2C and E).

Our method has been applied to the analysis of plasma samples from patients receiving hydralazine for treatment of severe pregnancy-induced hypertension. A representative chromatogram is shown in Fig. 3.

The results presented established the reliability and validity of the methodology. With the advent of the very sensitive electrochemical detector combined with the HPLC separation method, it has become feasible to extract, detect and quantify the salicylaldehyde hydrazone of hydralazine. Major advantages of the proposed method are its simplicity, sensitivity, accuracy, and specificity. Free hydralazine can be determined without interference from pyruvic acid hydrazone by using salicylaldehyde as a derivatizing agent. Finally, the method presented here can be recommended for routine patient monitoring or pharmacokinetic studies of unmetabolized hydralazine.

ACKNOWLEDGEMENTS

The authors wish to express thanks to Klaus Gerhardt and Charles Gehrke for their valuable suggestions on the manuscript, B. Skjonsby and K. Dorman for blood sample collection, and G. Dorhofer, W. Riess, and K. Scheibli (Ciba-Geigy, Basle, Switzerland) for donating 4-methylhydralazine and hydralazine pyruvic acid hydrazone.

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