Pharmaceutical Research, Vol. 5, No. 6, 1988

Report

Assay for Hydralazine as Its Stable *p*-Nitrobenzaldehyde Hydrazone

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Received October 1, 1987; accepted January 25, 1988

A new method of analysis for the antihypertensive drug, hydralazine, is introduced. The assay involves the addition of *p*-nitrobenzaldehyde to blood samples containing hydralazine, to form a stable Schiff's base, hydralazine *p*-nitrobenzaldehyde hydrazone. The derivative is extracted from the blood into hexane and the samples are dried under a nitrogen stream. The extracts are then dissolved in mobile phase and analyzed using high-performance liquid chromatography. The extracted samples can be stored for at least 7 days at room temperature or at −20°C. The sensitivity of the assay is better than 300 pg/ml using 3-ml blood samples, and the range can extend to 640 ng/ml. The stability of the extracted samples plus the sensitivity and simplicity of the assay are the main advantages of the method over other selective methods for hydralazine.

KEY WORDS: hydralazine; *p*-nitrobenzaldehyde derivatization; high-performance liquid chromatographic analysis of hydralazine; whole-blood analysis; hydralazine *p*-nitrobenzaldehyde hydrazone.

INTRODUCTION

Hydralazine is a peripheral vasodilator which has been widely used in the treatment of hypertension during the last 30 years. Pharmacokinetic studies have been hampered because many early assays lacked specificity and because the drug is very labile in plasma (1). Several recent specific high-performance liquid chromatographic (HPLC) assays for hydralazine in plasma have been published (2–6). All of these are complicated by requiring procedures for rapid cooling and/or separation of plasma immediately after withdrawing each blood sample. Ludden *et al.* (7) have used a simpler method, in which a Schiff's base between hydralazine and *p*-anisaldehyde is formed directly in blood. The hydralazine derivative is easily purified with a single hexane extraction prior to HPLC analysis. The assay is selective because the reaction is carried out at physiological pH, minimizing hydrolysis of acid-labile hydrazone metabolites which liberate derivatizable free hydralazine. The hydralazine *p*-anisaldehyde hydrazone derivative utilized in this assay has, however, been found to be erratically unstable on storage, even overnight at −20°C (8). In addition, the accuracy of hydralazine quantification in blood could be compromised because the internal standard derivative decomposed at a higher rate than the hydralazine derivative. In replicated samples where hydralazine quantification became a problem, a within standard variation of greater than 50% was not uncommon. A replacement for *p*-anisaldehyde was therefore sought, which would retain the selectivity and sensitivity of the assay but produce a more stable derivative. *p*-Nitrobenzaldehyde, which contains a strongly electron-withdrawing substituent, was used to produce a more stable Schiff's base. This report describes an HPLC assay of hydralazine as its *p*-nitrobenzaldehyde hydrazone, which can be stored in extracted samples for prolonged periods without decomposition.

MATERIALS AND METHODS

Reagents and Chemicals

Acetonitrile and hexane were HPLC-grade solvents (Fisher Scientific Co., Fairlawn, N.J.). Methylene chloride was ACS grade (Anachemia, Montreal, P.Q.). Hydralazine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 4-methylhydralazine, hydralazine pyruvic acid hydrazone (Ciba-Geigy Co., Basle, Switzerland), triethylamine, phosphoric acid, 85% (Fisher Scientific Co., Fairlawn, N.J.), *p*-nitrobenzaldehyde, and deuterochloroform (Aldrich Chemical Co., Milwaukee, Wis.) were used as received. (1,4)-Dioxane was glass distilled before use.

Preparation of Hydralazine *p*-Nitrobenzaldehyde Hydrazone

To 150 ml methylene chloride were added 0.75 g *p*-nitrobenzaldehyde and 1 g hydralazine hydrochloride to give approximately equimolar quantities. Triethylamine (0.5 ml) was added to neutralize the hydrochloride and liberate the hydralazine free base. A drying tube was attached, and the mixture stirred overnight. The resulting orange solution was filtered and evaporated to dryness. The residue was dissolved in a minimum volume of warmed methylene chloride and cooled until recrystallized. The solution was removed by filtration and the crystals dried under vacuum at 80°C. The identity and purity of the product were verified using GC/MS, UV, NMR, and melting-point determination.

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0724-8741/88/0600-0383$06.00/0

Glassware Treatment

Glassware was washed in FL-70 (Fisher Scientific Co., Fairlawn, N.J.), rinsed in deionized, distilled water, and muffled at 500°C for 2 hr, except for volumetric flasks, which were cleaned with chromic acid.

Standard Curves

All standard solutions were prepared daily. Hydralazine HCl (61.4 mg) was dissolved in 0.01 *N* HCl to make 100 ml of stock solution containing 0.5 mg/ml of free hydralazine base. From dilutions of the stock solution, three standard solutions were prepared containing 5 ng/μl and 500 and 100 pg/μl in 0.01 *N* HCl. The internal standard solution was prepared by dissolving 100 μg of 4-methylhydralazine in 50 ml of 0.01 *N* HCl. The derivatizing agent was prepared by dissolving 100 mg of *p*-nitrobenzaldehyde in 1 ml of dioxane.

Blood samples were collected into 60-ml plastic syringes, using EDTA as an anticoagulant. Aliquots of 1 or 3 ml were dispensed into 16 × 150-mm borosilicate glass tubes. Derivatizing agent (20 μl), internal standard (25 μl), and an appropriate amount of hydralazine were added to each blood sample. The tubes were capped with PTFE-lined caps, vortexed vigorously for 15 sec, and allowed to stand for 10 min. The derivatives were extracted by adding 3 vol of hexane to the tubes and mixing the samples on a Labquake rotary mixer for 10 min. The samples were centrifuged at 1000*g* for 10 min and the supernatants transferred to 16 × 100-mm glass tubes. The hexane was evaporated under a gentle stream of nitrogen and the residues were stored capped at −20°C until analysis.

Chromatographic Conditions

Each extraction residue was dissolved in 150 μl mobile phase, and the entire sample injected onto a 150 mm X 4.6-mm id Ultremex 3CN (Phenomenex, Palos Verdes, Calif.) reversed-phase column. The aqueous portion of the mobile phase contained 0.1% phosphoric acid (v/v) and 0.1% triethylamine (v/v) in deionized, double-distilled water, pH 3.0. The mobile phase consisted of 63% acetonitrile in aqueous phase, pumped at a flow rate of 2 ml/min. Detection was at 365 nm. The HPLC instrumentation included a Model 6000 pump, U6K injector, and Model 440 UV absorbance detector equipped with a 365-nm filter (all from Waters & Associates, Milford, Mass.).

Animal and Human Studies

The application of this assay to both human and canine subjects was demonstrated by measuring hydralazine concentrations after giving a 50-mg oral dose of Apresoline 20 mg/ml Injection solution (2.5 ml), followed by 100 ml of H_2O to an 86-kg human volunteer and after dosing a 30-kg canine conscript with a 1-mg/kg Apresoline injection iv (1.5 ml).

Blood samples (1 or 3 ml) were collected from the subjects into 3-ml plastic syringes whose hubs were filled with 1.0 *M* EDTA (approximately 0.05 ml). The syringes were quickly filled, then immediately emptied into 16 × 100-mm glass tubes. The internal standard and derivatizing reagent were added and the tube was vortexed. The time from sampling to vortexing was about 30 sec. The samples were then treated as described for the standard samples.

Data Analysis

The standard curve was evaluated by linear regression analysis of peak area ratio vs concentration. The data are expressed as means ± SD ($N = 5$). The two-tailed Student's *t* test was used to test for differences between groups ($P = 0.05$). The data were fitted using LAGRAN (9).

RESULTS

Structure Confirmation for Hydralazine *p*-Nitrobenzaldehyde Hydrazone

The chemical identity and purity of the *p*-nitro derivative were confirmed by GC/MS and melting-point determination, UV, and NMR spectrometry. The major diagnostic MS fragments are summarized in Table I. The melting point of 234–236°C (d), the ultraviolet spectrum in the mobile phase with a wavelength of maximum absorption of 356.6 nm, and the proton NMR spectrum of hydralazine *p*-nitrobenzaldehyde hydrazone measured in deuterochloroform were in good agreement with previously published data (10). A trace amount of contaminating hydrazine detected with NMR did not appear to affect the HPLC procedures.

Chromatography

The retention time of hydralazine *p*-nitrobenzaldehyde hydrazone was 3.76 min and that of 4-methylhydralazine *p*-nitrobenzaldehyde hydrazone, 4.88 min (Fig. 1). Reagent blanks and reagent blanks containing internal standard showed no interfering peaks. In dog blood, the standard curve was linear over the range 300 pg/ml to 640 ng/ml with a coefficient of correlation of 0.992 for 3-ml samples (Table II). The mean relative standard deviation was 5.8% (range, 2.55–11.97%) with no loss of precision at lower concentrations. Human data were similar, with a correlation coefficient of 0.999. For 1- and 3-ml blood samples, the sensitivity was better than 1 ng/ml and 300 pg/ml, respectively. The detection limit (when the peak height reached three times the baseline noise) was 33 pg/ml for 3-ml blood samples. Over 95% of the derivatization occurred within 1 min; the reaction was complete at 5 min. The derivatization and extraction efficiencies were 96 ± 4.0 and 74 ± 2.8%, respectively, for a

Table I. Diagnostic Fragments from the Reaction Product of Hydralazine HCl and *p*-Nitrobenzaldehyde[a]

m/z	Relative abundance (%)	Structure
293	33	$M^{+\cdot}$
292	12.4	$[M\text{-}H]^+$
171	100	$[M\text{-}PhNO_2]^+$

[a] The instrumentation consisted of a Hewlett Packard Model 5710A gas chromatograph coupled to a Model 5981A mass spectrometer and a Model 5934A 21MX data system. The ionization voltage was 70 eV. A 30-m DB-1 column (J&W Scientific, Folsom, Calif.) was used for separation. The temperature program consisted of 4 min at 100°C, followed by a ramp of 16°/min to 280°C, at which temperature the column was flushed for 16 min. The injector and detector temperatures were both set at 250°C and the flow rate of helium carrier gas was 1 ml/min. The retention time of the reaction product peak was 18.6 min.

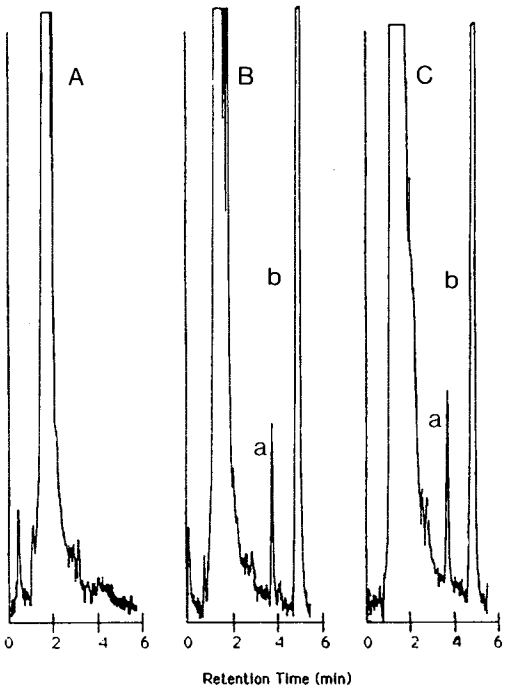

Fig. 1. Typical HPLC traces of *p*-nitrobenzaldehyde hydrazones of hydralazine (retention time = 3.76 min, a) and internal standard, 4-methylhydralazine (retention time = 4.88 min, b). (A) Reagent blank; (B) spiked 1-ml sample from standard curve, 2 ng/ml; (C) 1-ml sample from a dog 480 min after a 1-mg/kg iv dose of hydralazine HCl.

total recovery of 71 ± 1.9%. The selectivity of the new assay was verified by comparing apparent hydralazine recovery from pyruvic acid hydrazone using both the new and the *p*-anisaldehyde assays. When *p*-nitrobenzaldehyde and *p*-anisaldehyde were separately added to canine blood samples containing 3 μg of hydralazine pyruvic acid hydrazone, there was little difference between the two methods in the amount of apparent hydralazine detected (0.30 ± 0.026 vs 0.35 ± 0.024 mol%, respectively). None of the metabolites, 3-methyl-*s*-triazolo-[3,4-a]-phthalazine, *s*-triazolo-[3,4-a]-phthalazine, 4-hydrazino-phthalazin-1-one 4-(2-acetylhydrazino)-phthalazine, and phthalazin-1-one, produced peaks on the chromatograms.

Standard curves from freshly extracted samples and extracted samples stored for 7 days at room temperature or −20°C were compared. Both fresh, whole canine blood and citrated human blood were evaluated. The slopes from canine samples ± standard deviation (expressed as a percentage of the slope) were 0.0083 ± 7.1% for fresh, 0.0088 ± 7.2% for room temperature-stored, and 0.0089 ± 10% for −20°C-stored samples. From human samples, the slopes were 0.0103 ± 11.7% for fresh, 0.0099 ± 10.5% for room temperature-stored, and 0.0096 ± 9.7% for −20°C-stored samples. The slopes obtained from the different conditions did not differ from each other significantly.

Table II. Hydralazine Standard Curve Accuracy and Precision

Concentration added (ng/ml)	Concentration calculated (ng/ml)	Precision ±1 SD (%)
0.3	0.29	4.76
1	0.89	11.97
2	1.74	3.00
8	7.50	2.55
40	41	3.27
160	161	5.96
640	650	9.11

Figures 2 and 3 show blood concentration vs time data collected from the human and canine subjects. The curve for the human subject had a peak concentration of 7 ng/ml and a terminal half-life of 70 min. The dog concentration vs time data showed a biexponential decline with a terminal half-life of 151 min.

DISCUSSION

The advantages of the method of Ludden *et al.* are retained by the new hydralazine assay. The procedure is simple when compared with recently published assays for hydralazine, which require plasma separation plus one or more derivatization and extraction steps (2–6). Ludden *et al.* (2) reported that hydralazine pyruvic acid hydrazone, the major hydralazine metabolite in blood, was acid labile and its hydrolysis to "apparent hydralazine" could be minimized by derivatization at physiological pH. By using a similar reaction condition, we have found that *p*-nitrobenzaldehyde has no significant effects on the recovery of apparent hydralazine, indicating that the selectivities of the two assays are comparable. The new assay is superior to the *p*-anisaldehyde method because the hydralazine *p*-nitrobenzaldehyde hydrazone derivative provides an increase in sample stability, allowing prolonged storage of samples before analysis. The blood profiles of hydralazine in the dog using the two assays are shown in Fig. 3. Several aberrations can be seen in the curve produced from the *p*-anisaldehyde assay (Fig. 3B), and in our hands this profile could not be reproduced in the same dog on different occasions due to the instability of *p*-anisaldehyde hydrazone.

The new assay is three times more sensitive than the *p*-anisaldehyde assay so it is comparable to the more involved plasma assays using electrochemical detection (5,6). With these two improvements, the blood profile after an oral

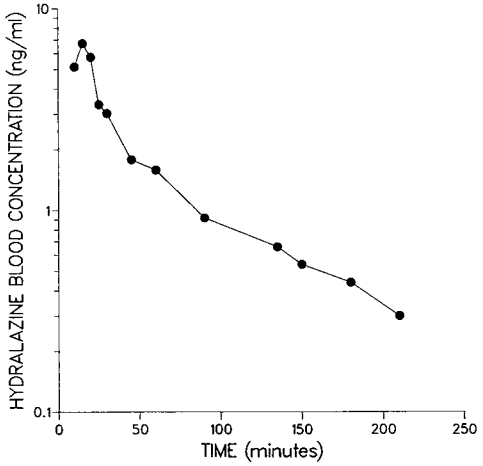

Fig. 2. Blood concentration vs time curve for an 86-kg human subject given a 50-mg oral dose of hydralazine.

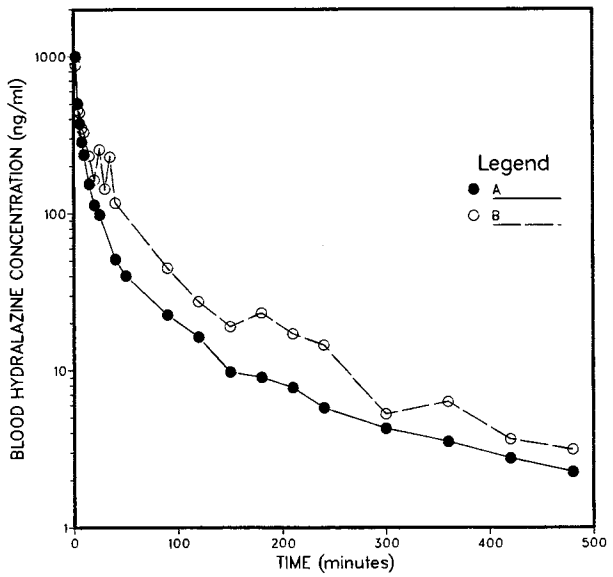

Fig. 3. Blood concentration vs time curves for a dog given a 1-mg/kg iv dose of hydralazine. (A) Using *p*-nitrobenzaldehyde as derivatizing agent and assay as described in the text; (B) using *p*-anisaldehyde as derivatizing agent and assay by the method of Ludden *et al.* (7).

dose can now be better characterized in humans. If a kinetic analysis were to be performed on the human curve (Fig. 2), using only the data above 1 ng/ml, the half-life would be 30.5 min, which is comparable to what has already been reported (11,12). Using the new method, however, a slower decline phase was discovered. This observation is consistent with the hypothesis of Ludden *et al.* (13) that the systemic elimination of hydralazine is limited by its return to the central compartment or by back conversion of labile metabolites. More studies are required to confirm this information.

The data presented here demonstrate the stability of the *p*-nitrobenzaldehyde hydrazones of hydralazine and 4-methylhydralazine on storage. This method provides a sensitive, selective, and simple procedure for the determination of hydralazine concentrations in whole blood and will be used to study the mechanism of hepatic extraction of hydralazine in the dog and human.

ACKNOWLEDGMENTS

This work was supported in part by the Alberta Heritage Foundation for Medical Research and the Alberta Heart Foundation. The authors thank Drs. G. Doerhoefer and K. Scheibli of CIBA-Geigy Co., Basle, Switzerland, for the generous donation of 4-methylhydralazine and hydralazine pyruvic acid hydrazone, Mr. Stephen Croteau for assistance with the GC/MS studies, and Dr. T. Hall for assistance with the NMR studies.

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