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ACKNOWLEDGMENTS

Presented in part at the Medicinal Chemistry Section, APhA Academy of Pharmaceutical Sciences, Phoenix meeting, November 1977.

Supported in part by a grant from the University of North Carolina Research Council.

High-Pressure Liquid Chromatographic Assay for Hydralazine in Human Plasma

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Received March 12, 1979, from the **Division of Clinical Pharmacology, Departments of Pharmacology and Medicine, University of Texas Health Science Center, San Antonio, TX 78284,* and the ‡*College of Pharmacy, University of Texas, Austin, TX 78712.* Accepted for publication June 4, 1979.

Abstract □ A specific high-performance liquid chromatographic assay for hydralazine in human plasma was developed. Plasma hydralazine is reacted with 10 μl of *p*-anisaldehyde for 7 min at room temperature to form hydralazine *p*-anisaldehyde hydrazone. This derivative is extracted into ethyl acetate, and the solvent is removed by evaporation. The residue is reconstituted in 100 μl of methanol, and 90 μl is injected onto a reversed-phase column. The mobile phase is 32% acetonitrile in 0.75 *M* acetate buffer, pH 3.4, at a flow rate of 2 ml/min. The retention time of hydralazine *p*-anisaldehyde hydrazone is 6.5 min. The average coefficient of variation over 10–200 ng/ml is 5.5%, and the sensitivity limit is 5 ng/ml. Under the assay conditions, hydralazine pyruvic acid hydrazone, a known plasma metabolite of hydralazine, yields <0.1% hydralazine. Detectable plasma hydralazine levels of 5–20 ng/ml were found 10–30 min after a 0.5-mg/kg oral dose of hydralazine hydrochloride was given to a male volunteer.

Keyphrases □ Hydralazine—extraction, high-pressure liquid chromatographic assay, human plasma □ High-pressure liquid chromatographic assay—hydralazine, human plasma □ Vasodilators—hydralazine, extraction, high-pressure liquid chromatographic assay

Hydralazine (1-hydrazinophthalazine, I) is a vasodilator used for treating hypertension (1). It undergoes extensive metabolism in humans, with detectable amounts of 3-methyl-*s*-triazolo[3,4-*a*]phthalazine, phthalazine, phthalazinone, 1-hydrazinophthalazine pyruvic acid hydrazone, 1-hydrazinophthalazine acetone hydrazone, 4-hydroxy-1-hydrazinophthalazine, and glucuronides (2–6) appearing in urine.

Previous attempts to assay I included derivatization with *p*-hydroxybenzaldehyde (7) or *p*-anisaldehyde (*p*-methoxybenzaldehyde) (8), followed by spectrophotometric determination or the conversion of I to tetrazolo[1,5-*a*]phthalazine followed by electron-capture GLC analysis (9, 10). All of these techniques are nonspecific in that acid-labile hydralazine hydrazones present in plasma, such as hydralazine pyruvic acid hydrazone, are hydrolyzed to some extent and detected as I (11).

This report describes a specific high-pressure liquid chromatographic (HPLC) assay for I in human plasma.

EXPERIMENTAL

Reagents and Chemicals—Ethyl acetate, acetonitrile, and methanol were purchased as glass-distilled solvents. No further processing was necessary. Hydralazine hydrochloride[1] and *p*-anisaldehyde[2] also were used as purchased. Hydralazine pyruvic acid hydrazone and hydralazine *p*-anisaldehyde hydrazone (II) were prepared as described previously (5) with the reaction mixture pH maintained at 7.4. The identity and purity of the synthesized products were verified by combined GLC–mass spectrometry. No by-products or starting materials were detected.

The absence of I was confirmed first by derivatizing samples of each synthetic product with bis(trimethylsilyl)trifluoroacetamide to form the *N,N'*-di-trimethylsilyl derivative of any I that might be present and then subjecting them to GLC–mass spectrometry. To demonstrate that I could be detected by this procedure, known amounts of I were added to samples of each synthetic product and assayed similarly. By this technique, it was demonstrated that the synthetic samples of hydralazine hydrazones contained <0.03% I.

Standard Curve Samples—Fresh human plasma, 1 ml, with ethylenediaminetetraacetic acid as the anticoagulant and 10 μl of *p*-anisaldehyde were placed in a 15-ml culture tube fitted with a polytef-lined screw cap. An appropriate volume, 0.5–20 μl (5–200 ng), of a fresh solution of 10 ng of I/μl of 0.1 *M* potassium phosphate buffer, pH 7.4, was added; the contents were mixed by vortexing.

After standing at room temperature (23°) for 7 min to allow formation of II, 1 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, and 7 ml of ethyl acetate were added. The samples were mixed for 10 min on a reciprocal shaker, and the phases were separated by centrifugation at 1000×*g* for 2 min. The ethyl acetate was transferred to a conical tube, and the solvent was removed in a gentle nitrogen stream.

Chromatography—Each extraction residue was mixed with 100 μl of methanol, and 90 μl was injected into a high-pressure liquid chromatograph[3] equipped with a reversed-phase column[4] (3.9 mm i.d. × 30 cm) and a variable-wavelength detector[5] set at 365 nm. The mobile phase was 32% acetonitrile in 0.75 *M* acetate buffer, pH 3.4, at a flow rate of 2 ml/min.

Derivatization and Extraction—The efficiency of derivatization was determined during three analyses by comparing the II recovery from 100-ng/ml standard hydralazine samples to the II recovery from blank plasma samples to which an equimolar concentration (173 ng/ml) of II had been added prior to extraction. Extraction efficiency was evaluated during three analyses by comparing the II peak height for plasma samples to which 173 ng of II was added before extraction to the II peak height for extracts of blank plasma samples to which 173 ng had been added just before injection into the liquid chromatograph.

Specificity—To assure that I does not react with an endogenous

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Model 995, Tracor, Austin, Tex.
[4] μBondapak C_{18}, Waters Associates, Milford, Mass.
[5] Model 970, Tracor, Austin, Tex.

0022-3549/79/1100-1423$01.00/0

Table I—Intraassay Precision of Hydralazine Assay

Hydralazine Added, ng/ml	n	Relative Peak Height[a]	Hydralazine Found, ng/ml
10	3	15.3 ± 1.53	9.1
50	4	79.8 ● 2.22	52.7
100	4	146 ± 5.69	97.8
200	4	298 ± 15.5	200

[a] Mean ± *SD*.

plasma component to yield a substance with a retention time identical to that of II, 5 μg of I/ml was added to plasma without *p*-anisaldehyde and taken through the procedure.

Hydralazine pyruvic acid hydrazone, a metabolite of I found in plasma, was added at 20 μg/ml to blank serum containing 10 μl of *p*-anisaldehyde and immediately taken through the assay.

Precision—Intraassay precision was evaluated by preparing and assaying multiple standard samples at 10, 50, 100, and 200 ng/ml. Intersubject differences on a given day were evaluated by preparing 10-, 50-, and 100-ng/ml standard samples using plasma from three human volunteers. Interassay precision was evaluated by comparing the slopes and intercepts of standard curves for different days.

Human Volunteer—A 29-year-old, 66-kg, male volunteer, known to be a slow acetylator, was given 33 mg of hydralazine hydrochloride orally. Blood samples were obtained at 0, 5, 10, 20, 25, 30, 40, 50, and 60 min. Blood was drawn into evacuated tubes containing ethylenediaminetetraacetic acid as an anticoagulant. The red cells were separated by centrifugation for 5 min at 1000×*g*.

Plasma, 1 ml, was then added to a culture tube containing 10 μl of *p*-anisaldehyde and vortexed. The interval between the midpoint of blood drawing and the mixing of plasma with *p*-anisaldehyde was kept between 6.5 and 8.0 min. Unknown samples were then treated exactly like the standard curve samples described earlier. Apparent plasma hydralazine levels also were determined by a modified (11) GLC procedure, similar to that reported by Jack *et al.* (9).

RESULTS

The retention time for II was about 6.5 min with the chromatographic conditions used. Occasional adjustment of the acetonitrile concentration between 31 and 33% was necessary to obtain optimal separation of the II peak from an extraneous peak (Fig. 1). Maximum sensitivity was 3–5 ng/ml, depending on column age.

Derivatization efficiency was 91.2 ± 7.8% (n = 3), while extraction recovery was 80.4 ± 9.3% (n = 3). These determinations were performed on 3 days.

After addition of hydralazine pyruvic acid hydrazone, 20 μg/ml, to blank plasma standards, there was only 8 ng of I/ml (0.06 mole %). Addition of I, 5 μg/ml, to plasma without *p*-anisaldehyde did not yield chromatographic peaks under the described conditions.

The results of the intraassay precision study are shown in Table I. The mean coefficient of variation was 5.5%. There were no differences in the coefficients of variation of 10-, 50-, and 100-ng/ml standards prepared in plasma from three volunteers. However, the slope and intercept of the standard curve varied somewhat from day to day: slope, 1.28 ± 0.135; and intercept, 0.15 ± 3.97 (mean ± *SD*). A daily standard curve was assayed with each set of unknowns.

Unchanged I could be detected transiently in the plasma of the human volunteer after an oral hydralazine hydrochloride dose of 0.5 mg/kg (Table II). The values obtained for apparent hydralazine using the GLC

Table II—Plasma Hydralazine Levels in a Human Volunteer after Oral Administration of 0.5 mg/kg

Minutes	Unchanged Hydralazine Concentration, ng/ml[a]	Apparent Hydralazine Concentration, ng/ml[b]
5	<5.0	50
10	14.4	210
20	19.6	250
25	11.5	240
30	11.2	227
40	<5.0	195
50	<5.0	172
60	<5.0	150

[a] Determined by described HPLC procedure. Concentration was at 6.5–8 min after blood drawing. Sensitivity limit was 5.0 ng/ml. [b] Determined by a modification (11) of a commonly used GLC procedure (9).

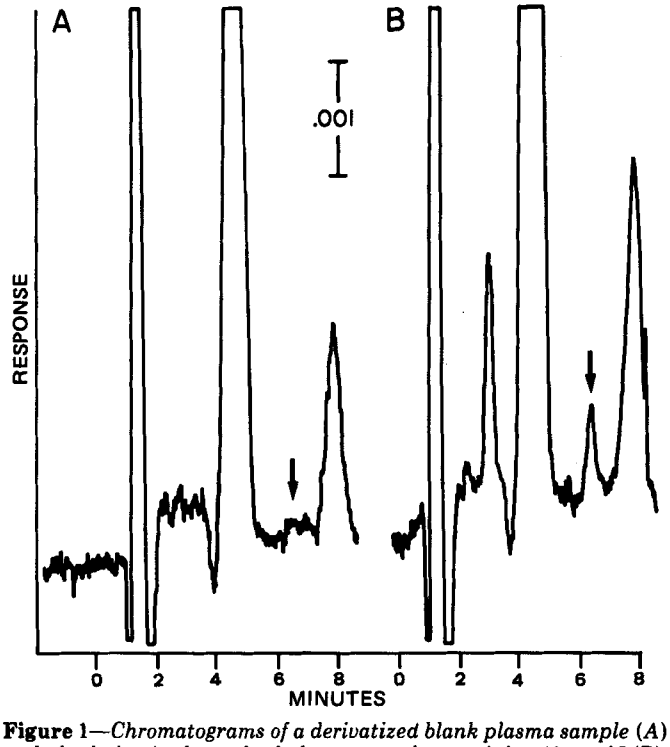

Figure 1—*Chromatograms of a derivatized blank plasma sample (A) and of a derivatized standard plasma sample containing 10 ng of I (B). The arrows indicate the retention time of II. The peak at 3.5 min in B appeared sporadically and was not related to the presence of I.*

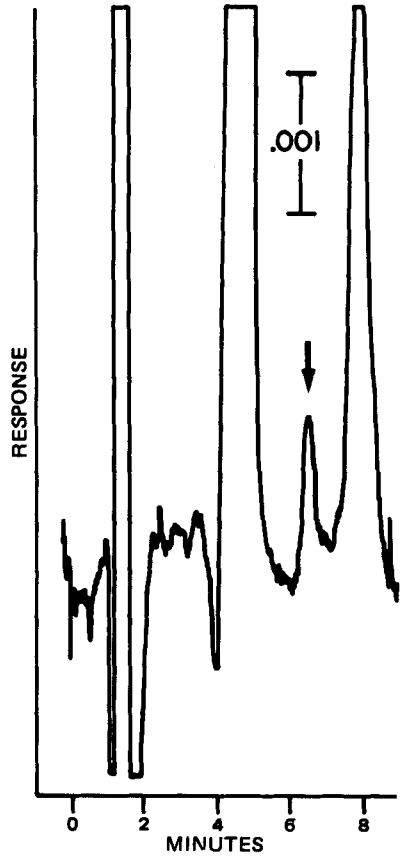

Figure 2—*Chromatogram of a derivatized plasma sample obtained 10 min after an oral dose of hydralazine hydrochloride, 0.5 mg/kg, to a human volunteer. The arrow indicates the retention time of II.*

procedure were considerably higher (Table II). The high-pressure liquid chromatogram of the 10-min sample is shown in Fig. 2.

DISCUSSION

The spectrophotometric assays that were used previously to measure the *p*-hydroxybenzaldehyde (7) or *p*-anisaldehyde (8) derivatives of I are nonspecific. These procedures used heat and acid catalysis to form the hydrazone derivatives of I in plasma. Under these conditions, acid-labile hydrazones of I, such as hydralazine pyruvic acid hydrazone, break down to I (10). Likewise, the GLC procedure of Jack *et al.* (9), which requires the formation of tetrazolo[1,5-a]phthalazine from I using nitrous acid (pH < 1), converts acid-labile hydrazones to free I.

Zak *et al.* (10) modified the GLC procedure so that the pH for tetrazolophthalazine formation was about 2.85 instead of <1. These investigators found considerably lower values for the concentration of "apparent" hydralazine in plasma than were reported previously. However, stability studies of hydralazine pyruvic acid hydrazone (11) indicated that this hydrazone is unstable at acid pH values. The plasma I levels found following a 0.5-mg/kg oral dose of hydralazine hydrochloride to a human volunteer using the described HPLC procedure are considerably lower than would be expected from previous studies, which used less specific analytical techniques (7–10, 12–14).

The described procedure avoids the use of acid or heat for formation of II in plasma. Authentic hydralazine pyruvic acid hydrazone, a major circulating metabolite of I in humans (15), yields <0.1 mole % of I when taken through the assay. Additional information about the circulating levels of other labile hydrazones is needed to assess method specificity.

Studies of the *in vitro* disappearance of I in plasma at 23° indicated a first-order process with a mean half-life of 16.1 min (16). Additional stability studies, carried out *in vitro* using whole blood and plasma, indicated that the disappearance of I is quite rapid at 37° (half-life ~6–10 min) but that loss during sample processing can be retarded by cooling in an ice water bath and centrifuging at 4°[6] (half-life ~1–2 hr). In the current study, blood samples from the volunteer were processed at room temperature, with the time between blood drawing and the addition of *p*-anisaldehyde to plasma held at 6.5–8.0 min. As much as 50% of I in plasma at the time blood was drawn could have been lost during processing. Even if the losses of I during processing were this high, apparent hydralazine levels (Table II) would still be fivefold or more higher than the concentration of unchanged hydralazine.

Although not sensitive enough for detailed pharmacokinetic studies following usual oral doses, the current assay can be applied to stability studies of I and its hydrazones in biological fluids and to pharmacokinetic studies following intravenous I administration to animals and humans (15). In addition, it can be used to monitor peak plasma levels of I in patients receiving chronic therapy.

[6] Unpublished data.

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ACKNOWLEDGMENTS

Presented as a poster session at the 63rd Annual Meeting of the Federation of American Societies for Experimental Biology, Dallas, Tex., April 1979.

Supported in part by U.S. Public Health Service NIH Grant GM24092.

The authors thank Ms. Diana Resendez and Ms. Barbara Boyle for their help in preparing this manuscript.