Development of Radioimmunoassay for Guanethidine

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Abstract □ A radioimmunoassay was developed for measuring plasma concentrations of the antihypertensive agent guanethidine at the nanogram level. Guanethidine was conjugated covalently to human serum albumin by two procedures, and the degree of conjugation was determined using tracer amounts of ^{3}H-guanethidine. Immunization of sheep with various conjugates afforded antiserums with specificity for guanethidine, as determined in competitive binding studies using ^{3}H-guanethidine and a dextran-coated charcoal technique for the separation of free and antibody-bound drug. The major human metabolites, an *N*-oxide and a ring-opened derivative, were not cross-reactive in antibody binding studies. Constituents of human plasma or serum do not appear to interfere with the assay. Preliminary results from immunoassay of plasma samples from patients receiving guanethidine indicate potential use for assessing dosage regimens and studying pharmacokinetics.

Keyphrases □ Guanethidine—radioimmunoassay, human plasma, nanogram level □ Antihypertensive agents—guanethidine, radioimmunoassay, human plasma, nanogram level □ Radioimmunoassay—guanethidine, human plasma, nanogram level

A need exists for an improved quantitative assay for the antihypertensive guanethidine[1] (I). Such a method should be simple, inexpensive, and clinically applicable with high sensitivity (nanogram or picogram level) and specificity. A radioimmunoassay could fill this need, making possible improved clinical monitoring of patient dosage regimens and the acquisition of pharmacokinetic data, the analysis of which could lead to more effective therapy.

BACKGROUND

A literature review (1–8) indicated that pharmacokinetic information concerning the absorption, distribution, and elimination of orally administered guanethidine sulfate is conflicting and has limited clinical applicability. Difficulties in applying this research to patient care may stem from insufficient assay sensitivity (and, in some cases, specificity), which has precluded the evaluation of samples containing very low guanethidine concentrations. Conflicting data may have arisen because plasma levels were not sampled over an adequate period.

Bioavailability studies comparing the usual oral dosage form with drug administered intravenously or as an aqueous oral solution have not been completed. Loading dose regimens for guanethidine have been developed (8–11). These efforts were in patients who did not demonstrate remarkable renal insufficiency. This previous research did not consider the influence of renal function on the drug disposition, and the pharmacokinetic parameters used in those dosage regimens may have been derived empirically.

In assessing the relationship between the guanethidine dose and the hypotensive response, Walter *et al.* (12) utilized the most definitive analytical technique for guanethidine (13); it determines guanethidine in biological fluids by GLC with flame-ionization detection or multiple-ion detection (mass spectrometry). Although this assay is specific and sensitive to concentrations as low as 1 ng/ml, the complexity of the manipulations (extractions and hydrolyses) and the limited availability and expense of the assay equipment (GLC–mass spectrometry and its operation) reduce its applicability in clinical studies.

Other methods for guanethidine appear to lack the desired sensitivity (and, in some cases, the specificity) for clinical studies. Briefly, these methods involve preliminary extraction and/or chromatographic procedures followed by quantitation *via* colorimetry (14–18) or fluorometry (19–21) and are not useful below microgram or milligram levels. The use of radiolabeled drug affords sensitivity to nanogram or picogram quantities (22); however, specificity for the drug and metabolites is not achieved.

Any analytical procedure for guanethidine must enable its determination in the presence of known metabolites and other interfering substances. The literature on guanethidine metabolism was reviewed recently (23), particularly the work of four research groups (19, 23–25). In two of the studies, direct metabolites were identified, the first involving ring *N*-oxide formation and the second involving ring α-hydroxylation followed by hydrolysis to a ring-opened amino acid (Scheme I, Structures II and III).

This paper describes the development of a sensitive and specific radioimmunoassay for guanethidine and some initial results on its clinical application.

EXPERIMENTAL

Materials—The following were used: human serum albumin[2] (crystalline, B grade); bovine serum albumin[2] (crystalline, A grade); guanethidine sulfate[3]; Freund's complete adjuvant[4]; dextran[5]; charcoal[5]; formalin solution[6]; commercial liquid scintillation fluid[7]; guanethidine metabolites 2-(octahydro-1-azocinyl-1-oxido)ethylguanidine[8] ("guanethidine-*N*-oxide"), 2-(6-carboxyhexylamine)ethylguanidine[8] ("ring-opened metabolite"), and 1-(6-carboxyhexyl)-2-iminoimidazolidine (a possible artifact arising from ring closure of the ring-opened metabolite)[8]; and ^{3}H-guanethidine[8] of low specific activity (62 mCi/mmole) (labeled on the C-2 of the ethyl group) (26).

Tritiated material of high specific activity (~300 mCi/mmole) was prepared commercially[9] and purified as described later. Liquid scintillation counting was accomplished using a spectrometer[10] with internal standardization to compute efficiency and disintegrations per minute in individual samples.

Coupling of Guanethidine to Human Serum Albumin (Scheme II)—Two methods involving formaldehyde as a coupling reagent were employed, one in glacial acetic acid and the other with sodium acetate in an aqueous medium (27). To 2.0 g of guanethidine sulfate in 20 ml of water was added 4 ml of ethanol containing approximately 2×10^6 cpm of ^{3}H-guanethidine[8]. The solution was evaporated *in vacuo* to dryness. After addition of 6 ml of cold 30% KOH, the free base was extracted into 5 × 50 ml of chloroform. The combined extracts were dried over magnesium sulfate and evaporated, affording 0.40 g of free base, which was stored under nitrogen.

Coupling Method A—To 82 mg of free base in 3.6 ml of glacial acetic acid was added 400 mg of human serum albumin in 4 ml of distilled water. Formaldehyde solution (38%), 2.4 ml, was added to this clear mixture. The mixture was heated and stirred at 50° under nitrogen for 1 hr and then stirred overnight at room temperature. The resulting clear solution was dialyzed *versus* several portions of distilled water until negligible radioactivity was detected in the dialysates. Scintillation counting of both dialysates and bag contents indicated 5.8 moles of guanethidine bound/mole of serum albumin based on a molecular weight of 70,000. The product was lyophilized to a white powder and stored in the cold.

Coupling Method B (28)—To 200 mg of human serum albumin in 2 ml of distilled water was added 2.0 ml of 3 *M* sodium acetate. Formaldehyde solution (38%), 4.0 ml, was added, and the mixture was stirred for 5 min at room temperature. A solution of 28 mg of guanethidine (free base) in 1 ml of water was added. The mixture was stirred under nitrogen

[1] Guanethidine sulfate (Ismelin), Ciba-Geigy Corp., Summit, NJ 07901.

[2] Calbiochem Corp.
[3] Ciba-Geigy Corp., Summit, NJ 07901.
[4] Difco Laboratories.
[5] Radioimmunoassay grade materials, Schwarz/Mann.
[6] J. T. Baker Chemical Co.
[7] Scintaverse, Fisher Chemical Co.
[8] Gift of Dr. D. F. Elliott, Ciba Laboratories, Horsham, England.
[9] New England Nuclear.
[10] Model 3255, Packard Instrument Co., Downers Grove, Ill.

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Table I—Data for Typical Guanethidine Standard Radioimmunoassay Curve

Tube	Buffer[a], ml	Bovine Serum Albumin[b], ml	Antiserum[c], ml	Guanethidine (Cold)[d] Milliliters	Guanethidine (Cold)[d] Nanograms	^{3}H-Guanethidine[e], ml	Dextran-Coated Charcoal[f], ml
1	1.30	0.10	—	—	—	0.10	—
2	0.80	0.10	—	—	—	0.10	0.50
3	0.70	0.10	0.10	0	0	0.10	0.50
4	0.68	0.10	0.10	0.02	2.0	0.10	0.50
5	0.66	0.10	0.10	0.04	4.0	0.10	0.50
6	0.62	0.10	0.10	0.08	8.0	0.10	0.50
7	0.50	0.10	0.10	0.20	20.0	0.10	0.50
8	0.60	0.10	0.10	0.10[g]	100.0	0.10	0.50

[a] Buffer was 0.15 *M* NaCl and 0.01 *M* sodium phosphate, pH 7.4. [b] Crystalline bovine serum albumin 2× (Armour). [c] Guanethidine antiserum was diluted 1:60 with buffer (final dilution 1:600 in tube). [d] Guanethidine (cold) was 0.10 ng/μl in buffer (used 2–100 ng in curve). [e] ^{3}H-Guanethidine was 0.1 ng/μl in buffer (12 ng/tube, ~1500 dpm). [f] Dextran-coated charcoal suspension contained 0.01 g of immunoassay grade charcoal per milliliter and 0.25 mg of dextran (immunoassay grade) per milliliter. [g] Concentration was 1.0 ng/ml in buffer.

at room temperature, dialyzed, and lyophilized as in Method A. Counting of tracer ^{3}H-guanethidine indicated 33 moles of guanethidine bound/mole of serum albumin.

Immunization Procedure and Schedule—Each guanethidine–albumin conjugate was injected intramuscularly (two sheep per conjugate) at several injection sites (shoulders and flanks) at monthly intervals. Initial and subsequent injections consisted of 3 mg of conjugate in 1.5 ml of physiological saline emulsified with 1.5 ml of Freund's complete adjuvant. Bleedings were made from the neck vein approximately 7–10 days after each monthly booster injection. To date, three of the four sheep have been boosted and bled for 18 months. Serum was frozen after the addition of thimerosal (final concentration, 0.01%) as an antibacterial.

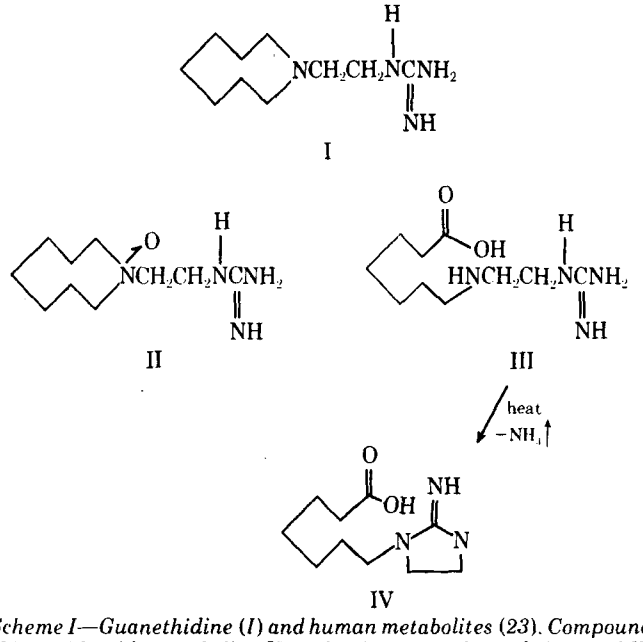
Titer Determination—Individual serum samples were evaluated by serial dilution for their ability to bind 12 ng of ^{3}H-guanethidine in the absence of added nonradioactive guanethidine. For this evaluation, 100-μl samples of antiserum dilutions were used as described later. In routine work, the antiserum dilution giving 40–60% binding was optimum.

Standard Radioimmunoassay Curve for Guanethidine—Table I indicates the order of reagent addition and the contents of various triplicate assay tubes utilized in the construction of a typical standard radioimmunoassay curve. Polystyrene test tubes (5 ml, 12 × 75 mm) were used. Bovine serum albumin (0.5%) was added to the buffer to minimize adsorption of ^{3}H-guanethidine[8] (62 mCi/mmole) and cold guanethidine onto the vessel walls, although no evidence for such adsorption had been observed. Antiserum was freshly diluted from stock on the day of use.

Following the antiserum addition, various quantities of cold guanethidine and 12 ng of ^{3}H-guanethidine (~1500 dpm) were added to each tube. The tubes were incubated at 37° for 2 hr and chilled in ice for 30 min. Then 0.5 ml of dextran-coated charcoal suspension was added to the desired tubes (Table I, footnote *f*). After vortexing, suspensions were left at 0° for exactly 15 min and then centrifuged at 2° for 10 min.

Exactly 1.0 ml of the supernate was removed carefully by automatic pipet and counted in 10 ml of scintillation cocktail in a counting vial. Percent binding figures were calculated. Nonspecific binding (~3%) was obtained from Tube 2 figures and subtracted from the total percentage figures observed for all tubes. In specificity (cross-reactivity) studies, cold guanethidine was replaced with desired quantities of the test compound. In the assay of clinical samples, up to 0.1 ml of unknown plasma or serum was employed, with pooled control plasma in all tubes.

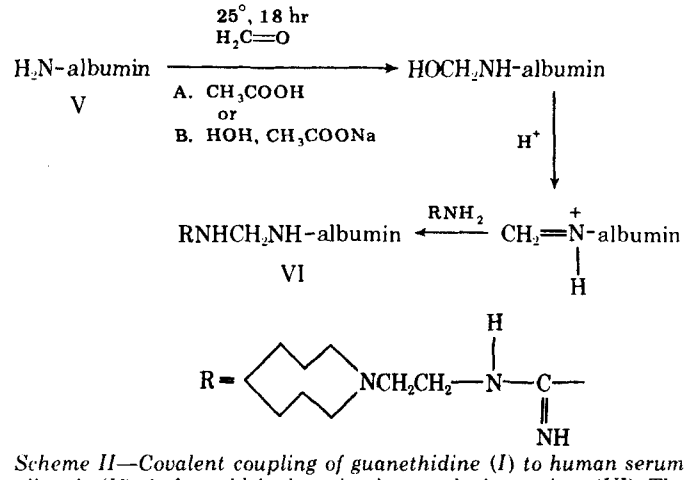
Synthesis of Randomly Labeled ^{3}H-Guanethidine of Increased Specific Activity—Guanethidine sulfate, 50 mg, was dissolved in 0.3 ml of dry dimethylformamide and heated overnight at 80° with 100 mg of 5% rhodium-on-aluminum oxide catalyst and 25 Ci of tritiated water[9]. After removal of excess tritiated water, purification was accomplished as follows. Approximately 45 μCi (100 × 10^6 dpm) of crude material was dissolved in 200 μl of water and placed on a column of 1.0 g of Dowex 50× (acid form, 100–200 mesh, 4% cross-linked), packed in a Pasteur pipet. Upon elution with water in 1-ml fractions, 25% of the radioactivity was recovered in the first five fractions. The remainder of the material was eluted in one peak with 5 *N* HCl, collected in approximately 10 fractions. The peak fractions were checked for purity by TLC and paper chromatographic radioscan [solvent systems: 2-butanol–3% (v/v) ammonia (3:1), R_f 0.8; and *n*-butanol–acetic acid–water (40:10:50), R_f 0.3 (20)]. The product was estimated by isotope dilution to have a specific activity of 300 mCi/mmole, or five times that of material obtained previously[8].



Scheme I—Guanethidine (I) and human metabolites (23). Compound II is the N-oxide metabolite, III is the ring-opened metabolite, and IV is a probable artifact of the isolation workup, involving cyclization of III.

RESULTS AND DISCUSSION

Antigen and Antiserum Preparation—It appeared desirable in antigen preparation to utilize the terminal guanidino group of the hapten guanethidine for attachment to a protein carrier. This approach would leave unaltered immunodeterminant features near the metabolism sites, favoring the formation of antibody specific for guanethidine (I) and not the major metabolites, the *N*-oxide (II) and the ring-opened product (III) (Scheme I). Three coupling methods were employed successfully; how-



Scheme II—Covalent coupling of guanethidine (I) to human serum albumin (V) via formaldehyde activation, producing antigen (VI). The guanethidine–albumin ratio in Method A was 5.8; in Method B, it was 33.

Table II—Reproducibility in the Assay of Known Quantities of Guanethidine

Sample	Guanethidine Added, ng/ml	Guanethidine Found, ng/ml[a] Mean (n = 3)	CV, %
1	2.00	2.07	±6.4
2	5.00	4.68	±3.8
3	10.0	9.77	±7.8
4	20.0	19.8	±8.9
5	50.0	48.1	±3.3

[a] Each value is the mean of three individual samples and the coefficient of variation of individual samples from this mean ($SD \times 100$/mean). The guanethidine content of each sample was calculated from a percentage binding figure, and a standard curve was constructed from the means of samples run in triplicate.

ever, only the formaldehyde method produced antiserum of useful titer (Scheme II). Tracer quantities of ^{3}H-guanethidine were added to the reaction media to enable ready calculation of the ratio of drug to albumin in the conjugate. With formaldehyde in glacial acetic acid (Method A), this ratio was 5.8 moles of guanethidine/mole of albumin. With formaldehyde in water containing sodium acetate (Method B) (28), this figure increased to 33 moles of guanethidine/mole of albumin.

With a mechanism of coupling involving only lysine amino groups (tryptophan and imidazole also might be involved), the maximum ratio for albumin would be about 60. Two other coupling procedures were employed to move the hapten guanethidine moiety further away from the protein, perhaps improving titer and specificity. The first procedure, involving the reaction of guanethidine with succinic anhydride followed by water-soluble carbodiimide coupling of the newly generated terminal carboxyl group to albumin, resulted in a hapten to protein ratio of 4–5:1. The second procedure involved initial selective coupling of toluene-2,5-diisocyanate (29) to albumin at pH 7.4 (at the 5-position) and coupling of the more hindered 2-isocyanate group to guanethidine at pH 9.0. In this case, a hapten to protein ratio of 13 was obtained. Unfortunately, however, neither the succinic anhydride nor the diisocyanate conjugates elicited antiserum of useful titer with the animals and conditions employed.

Immunization of four sheep with the two formaldehyde conjugates (two sheep per conjugate) was accomplished as discussed under *Experimental.* All four sheep produced antibodies after 2–3 months, two of titer approximately 1:600. The highest titer serum was taken from the two sheep immunized with the conjugate of the lower hapten to protein ratio of 5.8. All sheep have now been bled monthly for 18 months, with the titer remaining essentially constant, dropping off slightly only in one animal. This relatively low titer probably could be increased considerably with radiolabeled guanethidine of higher specific activity, either a tritiated or an iodinated derivative (^{125}I). Antiserum stored frozen in small portions has shown no evidence of loss of guanethidine binding ability.

Standard Immunoassay Curve—Because of interest in radiolabeled material of higher specific activity (enabling increased sensitivity), exhaustive work has not been carried out to determine optimum assay conditions with available tritiated material. Nevertheless, the assay appears to be reproducible and directly clinically applicable for guanethidine concentrations above 4–5 ng/ml of plasma or serum.

The order of reagent addition and the contents of various triplicate assay tubes utilized in the preparation of a typical standard curve are indicated in Table I. Antiserum was freshly diluted from frozen stock serum on the day of use. Fresh solutions of ^{3}H-guanethidine and cold standard guanethidine also were prepared daily by dilution of more concentrated frozen stock solutions. The entire process, including the incubation and subsequent charcoal adsorption, was completed in ~4 hr.

Data for a typical standard immunoassay sigmoid curve are plotted in Fig. 1. Each medium of different composition was run in triplicate. The data in Fig. 1 include typical results for standard deviations of triplicate samples. Curves could be linearized *via* logit transformation procedures to extend the useful range of application and enable curve fitting and computer interpolation. However, this was not done routinely because the errors found by utilizing points at the logit curve ends were much larger than those encountered by diluting samples so that they fall within the central portion of the sigmoid curve. If assay conditions were controlled, the standard curve varied only slightly; however, at least five points on the curve were always incorporated into each day's work. No significant differences were observed by altering the incubation times between 1 and 4 hr at 37° or varying the time of incubation with dextran-coated charcoal between 15 and 30 min at 0°.

Table III—Recovery of Guanethidine Added (50 ng/ml) to Patient Serum Samples in Various Concentrations

Sample	Guanethidine, ng/ml Found in Original Sample[a]	Guanethidine, ng/ml Total Found	Recovery of Added Guanethidine[d], %
GC	57	106	98
ML	76	120	88
PL	16	62	92
PL	26	74	96
CM	14	61	94
CM	28	73	90
CM	29	80	102
CM	43	101	116

[a] Values are the averages of two separate assays done in triplicate. Percentage recovery figures, therefore, reflect errors in the original assay, errors in the addition step, and errors in the assay of the "spiked" sample. Whole serum samples were used, and all necessary dilutions were repeated after spiking. [b] Average deviation of recovery from 100% was −3%.

Nonspecific binding in the absence of antiserum amounted to about 3%, and this figure was subtracted from samples containing antiserum. The addition of up to 10% (0.1 ml/ml) pooled normal human serum or plasma to the assay tubes did not appear to alter the shape or position of the curve, nor did heparin, disodium ethylenediaminetetraacetic acid, or citrate in normal concentrations utilized for anticoagulation. Preliminary experiments also indicate that the assay is applicable to urine analysis.

Sensitivity—For blood guanethidine levels observed during patient loading protocols, conditions giving the curve shown in Fig. 1 were most convenient. The reliable concentration range with the ^{3}H-guanethidine (64 mCi/mmole) employed was about 2–20 ng in the assay. Since 0.10-ml plasma aliquots were used, this level corresponds to a direct measurement in the range of 20–200 ng/ml of plasma. Use of ^{3}H-guanethidine of specific activity 300 mCi/mmole (2.5 ng/assay tube) enabled an increase in sensitivity to about 4–5 ng/ml of plasma (lower limit of 0.4–0.5 ng in the assay). This curve is not shown but affords a useful range of 4–~50 ng/ml of plasma.

Walter *et al.* (12) reported therapeutic levels of guanethidine as assayed by the method of Hengstmann *et al.* (13) to be 8–17 ng/ml. Therefore, as it stands, the immunoassay sensitivity, while adequate for the measurement of levels observed during loading or during "normal" therapeutic control, is not adequate for detailed pharmacokinetic studies. Consideration of the literature and practical experience at North Carolina Memorial Hospital indicate that such a procedure could contribute significantly to pharmacokinetic studies if it were sensitive to levels below 1 ng/ml, perhaps 0.1 ng/ml. It is plausible that accurate mathematical (pharmacokinetic) statements describing guanethidine elimination from plasma of normal volunteers or hypertensive patients can be made, providing the terminal elimination phase can be evaluated until guanethidine concentrations decline to such levels (27). Efforts to increase assay sensitivity through synthesis of hotter tritiated material or a suitably iodinated (^{125}I) derivative are in progress.

Specificity—Studies of cross-reactivity and antibody specificity were carried out on major metabolites II and III and the isolation artifact IV (23); the concentrations at which these compounds reduced binding of ^{3}H-guanethidine (12 ng) were compared to the concentration of guanethidine (cold) that had the same effect. Approximately 1% cross-reactivity was observed with the *N*-oxide (II); less than 0.1% was observed with the ring-opened metabolite (III) and IV. Antihypertensive agents diazoxide, methyldopa, hydralazine, and furosemide showed less than 0.1% cross-reactivity. To date, all of these specificity studies have been performed on bleedings from one sheep, with three bleedings appearing to be essentially identical.

Assay Reproducibility and Recovery Studies (30, 31)—Table II indicates the reproducibility of the assay as measured in prepared standards (10% pooled human plasma), giving means and coefficients of variation for amounts found *versus* amounts actually added. Results are based on triplicate tubes for each unknown concentration. These results were derived by using a standard curve similar to Fig. 1.

Recovery studies were conducted using actual plasma samples. Sample guanethidine content was measured on several patients (Table III). Selected samples encompassing a wide concentration range were "spiked" with 50-ng amounts of standard guanethidine and reassayed for total guanethidine. Recovery, expressed as percent, indicates that drug added to each original plasma sample over a range of concentrations is recovered in quantities between 88 and 116% (mean = 97%).

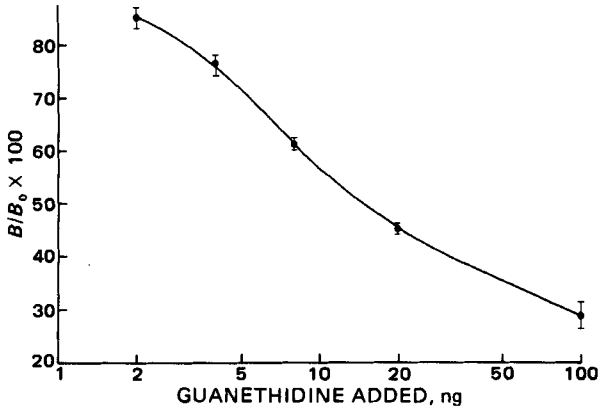


Figure 1—*Typical radioimmunoassay curve for guanethidine [serum dilution 1:600 (Sheep 37, 3-month bleeding), 12 ng of ^{3}H-guanethidine (62 mCi/mmole)]. Key: B_0, percent binding minus nonspecific binding in absence of cold guanethidine (maximum binding); and B, percent binding minus nonspecific binding in presence of cold guanethidine.*

Clinical Application during Patient Loading—Plasma concentrations have been monitored in several hypertensive patients receiving guanethidine as part of their antihypertensive regimen. Data on one patient are presented in Fig. 2. Patient CM was a 30-year-old male undergoing guanethidine loading. Guanethidine was given orally every 8 hr during the loading period as 50 mg of the sulfate. The therapeutic end-point was considered to be a standing diastolic (phase V) blood pressure in the 90–100-mm Hg range 8 hr after a 50-mg dose. In this patient, loading was reinstituted when concomitant hydralazine doses were halved and clinical effectiveness of the total therapeutic regimen diminished.

In Fig. 2, plasma guanethidine concentrations are shown superimposed on the total guanethidine dose administered during a single day. All determinations were made on plasma collected immediately prior to the administration of loading doses. Samples associated with maintenance doses were obtained approximately 26 hr following previous doses or, on two occasions, at 3 hr after the daily dose. Preliminary assessment of the utility of the analysis of plasma guanethidine concentrations suggests that monitoring during the administration of loading doses has limited interpretability. Variation in absorption from available tablets and, more importantly, the effects of the time necessary for complete tissue distribution may explain the observed fluctuations in plasma concentrations. Nevertheless, plasma level monitoring when steady state is approached represents a rational mechanism to assist in the evaluation of the clinical effectiveness of guanethidine. More complete data on patients over an extended time will be of interest.

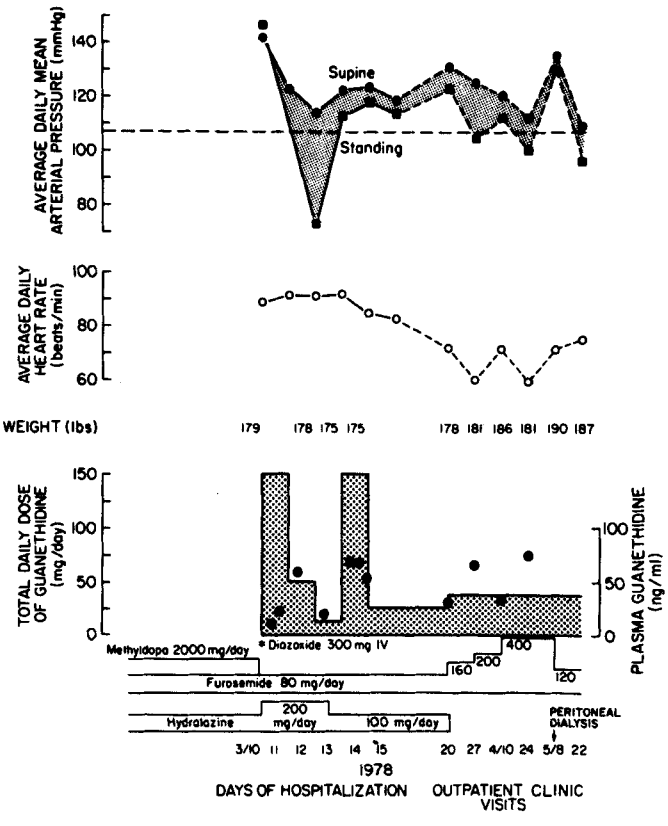


Figure 2—*Clinical application: response to guanethidine loading in a 30-year-old male with severe hypertension and renal insufficiency (Patient CM). Guanethidine levels in nanograms per milliliters are shown superimposed on lower bar graph (solid circles).*

Evaluation and Potential of Immunoassay Technique—In its present state, the assay described represents a rapid and direct method for the determination of plasma or serum guanethidine levels at concentrations encountered clinically without interference from major metabolites [which are inactive (23)], other antihypertensive agents, or common anticoagulants employed in blood collection tubes. Extractions or chromatographic purification procedures are avoided, and the determination is accurate to 4–5 ng/ml with an error of less than ±10%. Access to expensive equipment (*e.g.*, a mass spectrometer) with a skilled operator is obviated, with only scintillation counting instruments required.

Further work in progress using tritiated material of higher specific activity or a suitably labeled guanethidine analog containing iodine 125 should increase the titer, sensitivity, and convenience so that levels as low as 100 pg/ml might be measured directly. Such a convenient method should facilitate greatly the acquisition of accurate clinical data for research with the following goals: description of the relationship of plasma concentration to clinical effect (as measured by blood pressure), evaluation of bioequivalence and bioavailability of the drug from various dosage forms, description of the drug's pharmacokinetics, confirmation of the influence of renal function on guanethidine elimination from plasma, development and evaluation of loading dose regimens based on pharmacokinetic parameters, and overall provision of more rational methods for the selection of guanethidine dosages.

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High-Pressure Liquid Chromatographic Assay for Hydralazine in Human Plasma

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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.

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