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STUDIES ON THE METABOLISM OF GUANETHIDINE IN HYPERTENSIVE PATIENTS*

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Abstract—The urinary excretion of guanethidine (G) and its metabolites was studied in seven subjects with essential hypertension after a single oral dose of ^{14}C–G and after long-term treatment with oral doses ranging from 10 to 141 mg daily.

One metabolite (M_1) could be adsorbed on and eluted from cationic exchange resin in the same way as G itself. However, G was extractable into ether from alkaline solutions, whereas M_1 remained in the aqueous phase. Both G and M_1 were measured by a specific fluorometric method and by radioassay. The urine of patients treated with ^{14}C–G contained more radioactivity than could be accounted for by G and M_1, indicating the presence of at least one more metabolite of the drug.

ALTHOUGH guanethidine (G, structure in Fig. 1) is widely used as an antihypertensive agent, little information is available concerning its metabolism in man. Dollery *et al.*[1] did not find any metabolites of the drug in the urine of subjects who had received the labeled compound. These investigators, however, did not use a chromatographic method to separate G from possible metabolites. Metabolites of G have been reported in urine after administration of the drug to rats. Dollery *et al.*[1] found three urinary metabolites after the subcutaneous (s.c.) administration of G to that animal, but Bisson and Muscholl[2] and Furst[3] found only one metabolite in rat urine after intravenous administration of the drug. The present study was designed to study the metabolism of G in hypertensive patients, using effective separation methods and sensitive assay procedures.

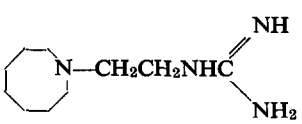

FIG. 1. Structure of guanethidine.

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METHODS

All measurements were carried out using urine from seven female subjects (B, C, E, H, J, K and M) with essential hypertension; their age ranged from 40 to 67 yr. All had normal liver function, as revealed by the standard tests, normal serum creatinine, and their creatinine clearance exceeded 60 ml/min. All medication except for digoxin (in the case of patients H, J, K and M) was discontinued for at least 1 week prior to the urine collections.

Urine was collected in plastic bottles containing enough sodium metabisulfite to give a final concentration of 0·5–3 mg/ml. The 24-hr excretion of creatinine in all urines did not differ by more than 40 per cent from the maximal daily creatinine excretion observed in a given patient.

Cation exchange resin (Bio-Rad Laboratories, Richmond, Calif., AG 50 W-X4, 200–400 mesh) was prepared for each urine sample as described by Bisson and Muscholl.[2] To an aliquot of 9 ml of filtered urine, 1 ml 10 N HCl was added. This mixture was allowed to pass through the column at a flow rate of 5 ml/30 min. Thereafter, the column was washed with 20 ml of 1 N HCl and 10 ml of 2 N HCl at a flow rate of 5 ml/15 min. No G could be detected in these fractions by fluorometric assay. Finally, 10 ml of 6·25 N HCl was passed through the column at a flow rate of 5 ml/30 min. From this fraction, 1 ml was used for fluorometric measurement. In trial runs involving the addition of 30–100 μg G to 9 ml of urine, 96 $\pm$ 1·8% [number of estimations (n) = 12] was recovered in the 6·25 N HCl eluate (F) from the ion exchange columns by fluorometric assay. The same recoveries were obtained by liquid scintillation counting if 30–100 μg G (3·14 mμc ^{14}C) was added to 9 ml of urine. All data reported herein are corrected for this per cent recovery.

In a 60-ml glass-stoppered bottle, 2·5 ml 20 N NaOH was added to 5 ml of F from ion exchange columns while cooling in an ice-bath. After adding 20 ml of ether, the mixture was shaken for 15 min in an automatic shaker. Upon centrifugation, an aliquot (10–15 ml) of the ether phase was evaporated to dryness. The residue was redissolved in 3 ml of 6·25 N HCl and 1 ml of this solution was used for fluorometric assay. Of 30–100 μg G added to 9 ml of urine, 80 $\pm$ 2·1 per cent (n = 12) was recovered from the ether phase by fluorometric measurement. Equivalent values for recovery were obtained by radiochemical methods.

G was measured fluorometrically using an Aminco–Bowman spectrophotofluorometer as described previously.[2] The excitation and fluorescence wavelengths were 400 mμ and 505 mμ (uncalibrated). Spectra were displayed on a Tektronix oscilloscope (type 502A) and photographed by means of a Polaroid camera. There was a linear relationship between fluorescence intensity and G concentrations ranging from 0·15 to 8 μg/ml. All measurements were done in duplicate. In control studies, internal standards were used besides external standards of 10 μg G. Since no quenching was observed, further fluorometric assays were carried out using external standards only.

Blank urine samples from each patient were collected prior to G treatment and for at least 2 weeks after G treatment was discontinued. When ion exchange chromatography only was used, the urine blank from the 7 subjects, as measured by fluorometric assay, corresponded to 3·2 $\pm$ 0·28 μg G/ml of urine. Ion exchange chromatography of urine samples followed by ether extraction from the alkaline solutions lowered the blank to 0·4 $\pm$ 0·028 μg G/ml of urine. Paper chromatography of F from ion exchange

columns was carried out as described,[2] using 2-butanol:3% (v/v) ammonia (3:1) as the solvent system (system A). Another solvent system used was the organic phase of *n*-butanol:glacial acetic acid: H_2O (40:10:50) (system B). Ether extracts were reduced *in vacuo* to give a volume of approximately 2 ml and spotted on paper. The chromatograms were developed (ascending fashion) for 15 hr on Whatman No. 1 paper, except where indicated otherwise. For visualization, the papers were sprayed with Dragendorff's reagent for alkaloids and subsequently with 0·1 N H_2SO_4.[4] Prior to use, the papers were washed with 0·01 N HCl by ascending chromatography.

Radioactivity was measured by a Beckman LS-250 liquid scintillation spectrometer. The scintillation solution was prepared as follows: 7 g 2,5-diphenyloxazole (PPO), 0·36 g 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl benzene (POPOP), was dissolved in 1000 ml of toluene, and 200 ml of solubilizer (Beckman Bio-Solv, BBS-3) was added. To 18 ml of this mixture, 1 ml urine or 2 ml ether was added in a counting vial. Aliquots of 0·1 to 1 ml of the eluates from the ion exchange columns were evaporated to dryness at room temperature; the residues were redissolved in 1 ml of a solution containing acetone:0·1 N HCl (2:1) and added to the scintillation mixture. Quenching was corrected for by the internal standard method, and the counting error was below 2 per cent. For the assay of chromatograms, squares of paper were placed at the bottom of the counting vials and 18 ml of scintillation mixture was added. Background (65 cpm) was subtracted from all values.

G* was always used as the sulfate; all data are expressed in terms of the base. The sample of ^{14}C-G* used (labeled in the guanidino-carbon) had a specific activity of 3·14 μc/mg. Its radiochemical purity was checked prior to use by paper chromatography in solvent system A. More than 95 per cent of the radioactivity had the same R_f value as authentic G. All data presented in the tables are in terms of mean $\pm$ standard error, with the number of estimation (n) in brackets.

RESULTS

Analysis of urine from human subjects during long-term treatment with G. Five hypertensive patients (C, E, H, J and M) were treated for at least 4 weeks with doses of G ranging from 10·0 to 141·0 mg per day. Thereafter, aliquots of a 24-hr urine specimen from each patient were used for ion exchange chromatography. Aliquots (5 ml) of F from subjects C, E, J and M were further analyzed by paper chromatography using solvent system A. After spraying with Dragendorff's reagent, the paper chromatograms of F from urine of the four subjects treated with G showed a bright red line corresponding to an R_f value of 0·9 $\pm$ 0·017, which was the same as that of authentic G. Another red line corresponding to an R_f value of 0·29 $\pm$ 0·005 (n = 4) was also seen in all these chromatograms (Fig. 2). No red line was seen after spraying with Dragendorff's reagent in chromatograms obtained from urine of patients in whom G treatment had been discontinued for at least 2 weeks. Eluates of paper strips corresponding to R_f values of 0·9 and 0·29 in solvent system A were rechromatographed in solvent system B and were both found to have an R_f value of 0·25. (Note that in solvent system B, authentic G, as well as M_1, had an R_f value of 0·25.) These results indicate the presence of G and of a single metabolite of the drug (M_1) in F obtained with ion exchange chromatography of urine.

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To make further characterizations, G and its metabolite (eluate from paper chromatograms, solvent system A) were reacted with ninhydrin[2] and their excitation and fluorescence spectra were recorded; it was found (Fig. 3) that the resulting spectra were indistinguishable from those of authentic G. There were two excitation peaks, 310 mμ and 400 mμ, and one fluorescence peak at 505 mμ (wavelengths uncalibrated).

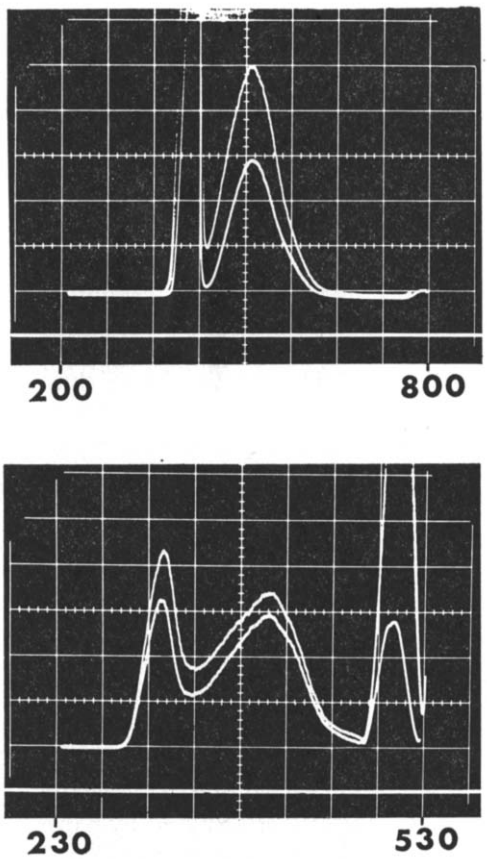

FIG. 3. Fluorescence (upper picture) and excitation (lower picture) spectra of authentic G (lower tracing) and a metabolite (M_1) (upper tracing) of the drug. The scales indicate wavelengths in mμ. The peaks to the left in the upper picture and to the right in the lower picture are due to light scattering.

F obtained by ion exchange chromatography of urine from patients J and M were extracted into ether as described under Methods, and the extracts were chromatographed on paper using solvent system A. There was one red line on these chromatograms corresponding to the R_f value of authentic G. No metabolite of G could be detected. Thus, combining ion exchange chromatography with ether extraction allows a differential estimation of G and M_1. Table 1 summarizes the results of differential estimation of G and M_1 in urine from the 5 patients studied during long-term treatment with the drug. The chemical structure of M_1 is not known at present, though being more polar it could be a hydroxy derivative. However, since the fluorometric assay

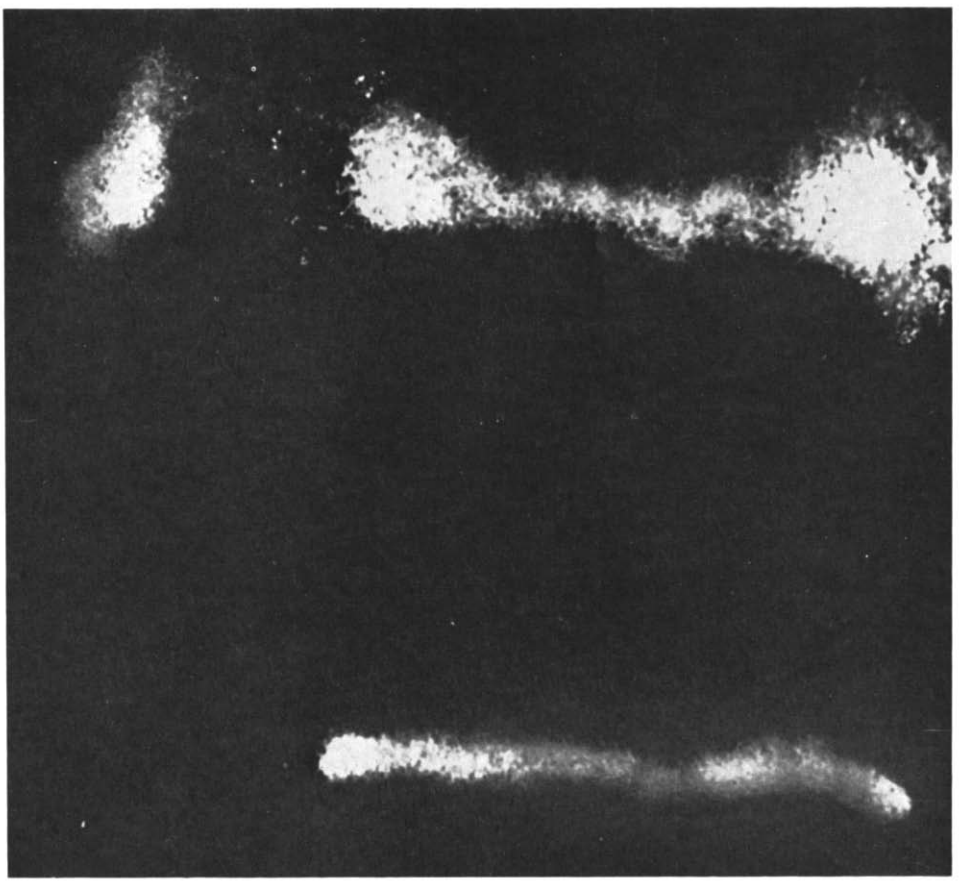

FIG. 2. Paper chromatogram of an eluate obtained by ion exchange chromatography of urine from patient J. The chromatogram was sprayed with Dragendorff's reagent for alkaloids and 0·1 N H_2SO_4. white line corresponds to an R_f of 0·9 which is the R_f of authentic G (white spot). The lower white line is due to M_1.

depends on the presence of the guanidino group,[5, 6] the data for M_1 are expressed in terms of G base. This is done on the assumption that equimolar concentrations of G and M_1 have the same fluorescence intensity when treated with ninhydrin. This assumption appears correct, based upon a comparison of fluorometric and radiochemical measurements using urine from patients treated with ^{14}C-G (see Table 3).

TABLE 1. URINARY EXCRETION OF GUANETHIDINE (G) AND ONE METABOLITE (M_1) IN SUBJECTS DURING LONG-TERM TREATMENT WITH THE DRUG*

Patient	Dose of G (mg/day)	Urinary excretion			
		mg/24 hr G	%†	mg/24 hr M_1	%†
H	10·0	0·54	5	0·58	6
C	32·2	2·55	8	1·13	4
E	40·4	2·02	5	1·11	3
J	121·0	3·06	3	7·09	6
M	141·0	10·62	8	2·83	2

* The amount of M_1 is given in equivalents of G base. Both G and M_1 were measured by fluorometric assay.
† The values given represent G and M_1, respectively, in per cent of the daily dose of the drug.

Analysis of urine from subjects treated with ^{14}C-G. Treatment with G was discontinued for at least 2 weeks in four subjects (B, H, J and K). Then after an overnight fast, they received a single oral dose of 41·6 mg G (5 μc). The urine collected during the following 12 hr contained enough radioactivity to allow chromatographic analysis. Aliquots of 1 ml urine (H and K) were evaporated to approximately 0·3 ml and this was chromatographed on Whatman No. 4 paper using solvent system A. Part of the radioactivity has a distinct R_f value of 0·85, corresponding to that of authentic ^{14}C-G. The rest of the ^{14}C in the urine was found in paper strips corresponding to R_f values from 0·1 to 0·5 without a clear peak being developed, indicating the possible presence of several metabolites.

Urines from subjects B, H and J were passed through an ion exchange column. The effluent which contained the radioactivity not adsorbed on the resin was collected, an aliquot was evaporated to dryness and counted after being redissolved in acetone:0·1 N HCl (2:1). Thereafter, the eluates were collected in 2·5-ml portions and aliquots of these also prepared for counting. Of the radioactivity present in the original samples, 47 $\pm$ 8 per cent was not adsorbed on the resin or was eluted by 1 N HCl (Table 2). Only 3 $\pm$ 0·2 per cent of the radioactivity was eluted by 2 N HCl, whereas 50 $\pm$ 5·1 per cent (n = 3) was found in F.

Aliquots (2 ml) of F (urine of subjects B, H, J and K) chromatographed on paper in solvent system A, showed a radioactive peak with an R_f of 0·84 $\pm$ 0·024 (n = 4); this R_f value is the same as that of authentic ^{14}C-G. Another ^{14}C peak was found in paper strips corresponding to an R_f of 0·26 $\pm$ 0·017 (n = 4) (Fig. 4). After ether extraction of F, there was only one radioactive peak, corresponding to the R_f of authentic G. These results confirm that G can be separated from M_1 by extraction into ether from alkalinized solutions of F. (Less than 10 per cent of M_1 was extracted into ether under these conditions.) An aliquot of 1 ml from the 1 N HCl eluate of

urine (subject B) was evaporated and redissolved as described and chromatographed in solvent system A. All the radioactivity was found in paper strips corresponding to R_f values from 0·03 to 0·5 with no distinct radioactive peak.

It was considered that the radioactive material not adsorbed by the resin might be chemically identical with M_1, i.e. part of M_1 not being adsorbed while passing through the column. To exclude this possibility, 5 to 9 -ml aliquots of effluent from the urine sample other than F were subjected to ion exchange chromatography again. Only 10 ± 2·2 per cent (n = 4) of the radioactivity not adsorbed from the urine samples during the first passage through the resin was retained and eluted by 6·25 N HCl. However, 94 ± 8·1 per cent (n = 2) of the 30 μg ^{14}C-G (3·14 mμc) added to the urine after the first ion exchange chromatography was recovered in F from the second chromatography. These results indicate that urine contains at least one more metabolite (M_2) which is not adsorbable on cationic exchange resin.

TABLE 2. ION EXCHANGE CHROMATOGRAPHY OF URINE FROM SUBJECTS TREATED WITH ^{14}C-G

Subject	B	H	J
Radioactivity	cpm × 10^3	cpm × 10^3	cpm × 10^3
Before ion exchange chromatography	37·5	9·90	44·9
Not adsorbed	10·6	1·97	11·3
1 N HCl eluate	9·58	1·32	11·2
2 N HCl eluate	1·14	0·22	1·31
6·25 N HCl eluate (F)	14·1	5·46	23·7
% Recovery	94·5	90·7	106

* Aliquots of 9 ml of urine were given on ion exchange columns. The effluent containing the ^{14}C not adsorbed on the resin was collected. The sum of ^{14}C found in the different fractions (last row) account for 97 ± 5·2 per cent of the total ^{14}C in the urine prior to ion exchange chromatography.

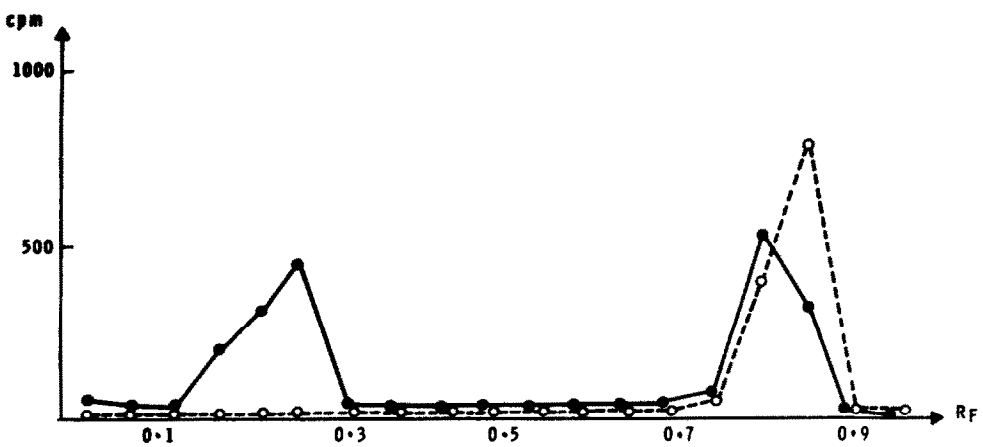

FIG. 4. Paper chromatogram of an eluate obtained by ion exchange chromatography of urine from subject J, ● — ●, and of authentic ^{14}C-G, ○ --- ○. Abscissa: R_f values. Ordinate: radioactivity (cpm) along the paper strips.

Table 3 gives the values for the urinary excretion of G and metabolites within 6 hr after an oral dose. Values by fluorometric assay are somewhat lower than the values by radiochemical assay. However, the results indicate that the M_1 can be estimated by the fluorometric method used.

Both G and M_1 are stable at 4° in urine and in F from ion exchange columns for at least 6 days. Furthermore, urine can be kept frozen for at least 2 weeks and can be thawed thereafter without change in the content of G and its metabolites.

TABLE 3. URINARY EXCRETION OF GUANETHIDINE (G) AND ITS METABOLITES AFTER A SINGLE ORAL DOSE OF ^{14}C-G

Patients	Urine volume (ml)	G (μg)		M_1 (μg)		M_2 (μg)
		Fluor.	Radioch.	Fluor.	Radioch.	Radioch.
B	160	880	1170	550	610	1490
H	150	880	1370	540	590	1590
J	150	990	1250	680	910	2100
K	900	1100	1370	1250	1410	3440
Average		960	1290	760	880	2160

* The subjects received 41·6 mg G (5 μc) and urine was collected during the following 6 hr. The values for G and its metabolites are expressed in μg base.

DISCUSSION

G is known to be metabolized by rat liver microsomes to a polar compound.[7] A metabolite of G which seems to be identical with this polar compound has been isolated from urine and various tissues of rats.[2, 3] Apparently, the same metabolite as described in rats by Bisson and Muscholl[2] and Furst[3] was found in our study for the first time in urine from hypertensive patients treated with G (M_1). M_1 is adsorbed on and eluted from cationic exchange resin in the same way as the metabolite isolated from rats. Both have similar R_f values in the same solvent system when subjected to paper chromatography, the same excitation and fluorescence spectra, and are not extractable into ether.

In studies of subjects treated with ^{14}C-G, there was more radioactivity excreted in urine than could be accounted for by G and M_1. Our data indicate that at least one more metabolite (M_2) is formed in man and excreted in urine. M_2 differs from M_1 in not being adsorbed on cation exchange resin. Bisson and Muscholl[2] could not detect this metabolite since these authors did not use labeled G, and they measured only the material adsorbed on cationic exchange resin. In the study of Furst,[3] who administered ^{14}C-G or ^{3}H-G to rats intraperitoneally, only one metabolite is mentioned. However, Dollery *et al.*,[1] who treated rats with subcutaneous doses of unlabeled G, identified three metabolites of the drug by paper chromatography of urine. The doses of G used in the study of Dollery *et al.*[1] and the study of Furst[3] were similar.

All three metabolites of G described by Dollery *et al.*[1] could be stained with reagents for guanidium compounds, suggesting that this group was still present in these metabolites. In our study, M_1 yielded a strong fluorescence when treated with ninhydrin, a reaction which is typical for compounds having a guanidino group.[5, 6] Because of high blanks, we were unable to measure M_2 by fluorometric assay.

G isolated from the urine of human subjects had the same fluorescence characteristics and the same R_f value when subjected to paper chromatography as authentic G, thus emphasizing the specificity of the methods used. The amount of M_1 excreted in urine in 24 hr may be more than twice as high as the amount of G during long-term treatment. Furthermore, the results show that a considerable fraction of the oral dose of G is excreted in urine as M_2 within 6 hr after administering the drug, indicating a rapid metabolism in man. Apparently, only minimal amounts of G and its metabolites are excreted into the bile in man and animals.[1, 8, 9] Furthermore, the radioactivity excreted in urine of subjects treated with oral doses of ^{14}C-G accounts for practically all the drug absorbed from the intestine.*

Acknowledgement—The careful technical assistance of Miss Nan Black is gratefully acknowledged.

Note added in proof—Since the submission of the present paper, several metabolites of G were structurally identified by C. McMartin (*Biochem. Pharmac.* **18**, 238 (1969)). One of these, the *N*-oxide could be M_1.

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