Keyphrases

Stability prediction—pharmaceuticals
5-Trifluoromethyl-2′-deoxyuridine—hydrolysis
pH effect—stability
Temperature effect—stability
Kinetic equations—degradation rates
Paper chromatography—degradation monitoring
UV spectrophotometry—degradation monitoring

Metabolism of β-Phenethylbiguanide

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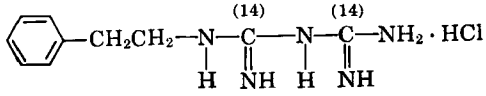
Carbon-14 labeled β-phenethylbiguanide (phenformin) was administered orally to male Sprague-Dawley rats. The radioactive material excreted in the urine was isolated and analyzed. The metabolic products were divided into "weak" and "strong" base components by their behavior on acidic ion-exchange resins. Chromatographic and spectroscopic evidence confirms the structure of the strong base fraction to be ρ-hydroxy-β-phenethylbiguanide. This hitherto unreported biguanide has been prepared synthetically for comparative purposes. The weak base metabolite has been shown to be the glucuronic acid conjugate of the phenolic biguanide. This fact was determined by acid hydrolysis to ρ-hydroxy-β-phenethylbiguanide and glucuronic acid. The more specific hydrolysis by β-glucuronidase confirmed the conjugate structure. The hydroxylated β-phenethylbiguanide along with its glucuronide conjugate were found to be present in the urine in approximately equal amounts. These two compounds seem to be the major metabolic products of β-phenethylbiguanide.

In diabetic humans, β-phenethylbiguanide hydrochloride has a hypoglycemic effect. Although this effect has been extensively studied, there is no unified concept which will explain the hypoglycemic action of this drug. Since the physiological activity of a chemotherapeutic agent is dependent in many cases (1) upon the mode of metabolism of the drug, an understanding of the metabolism of β-phenethylbiguanide may be helpful in elucidating the site and mode of action of the drug.

Preliminary studies in these laboratories have shown that the radioactivity of the labeled drug after administration is initially concentrated in the liver and gastric juice and is almost entirely eliminated in a 24-hr. period (2). Subsequent studies showed that the excreted metabolic products were chemically different from the ingested compound (3). The purpose of this study was the elucidation of the structure of the metabolic products excreted after the administration of β-phenethylbiguanide hydrochloride.

EXPERIMENTAL METHODS

In order to facilitate the metabolic studies, radioactive β-phenethylbiguanide hydrochloride synthesized by Walton (4), was used. The carbon-14 label was in the biguanide nucleus as depicted in the following:



Radioactivity was determined by the following means: (*a*) when the specific activity was to be determined the sample was oxidized to carbon dioxide by the "wet oxidation" method of Van Slyke and Folch (5) and precipitated as barium carbonate. The barium carbonate samples were mounted on planchets and counted for radioactivity in a Nuclear-Chicago gas-flow counter; (*b*) when relative radioactivity was desired, as in the case of column chromatography, aliquots were placed directly on the planchets, evaporated, and counted for radioactivity.

All paper chromatography was done by ascending technique on Whatman No. 1 paper. The spray

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used for the detection of biguanide metabolites was prepared 30 min. prior to use by mixing 10% solutions of sodium nitroprusside, potassium ferricyanide, and sodium hydroxide in a 1:1:1 ratio and diluting the solution with 9 vol. of water. The reagent gives a bright red color with guanidines and biguanides (6).

Ultraviolet spectra were run on a Cary model 14 recording spectrophotometer. Infrared spectra were run in potassium bromide pellets on a Perkin-Elmer Infracord spectrophotometer. Nuclear magnetic resonance spectra were obtained on a Varian A-60 spectrometer.

ISOLATION OF METABOLIC PRODUCTS

β-Phenethylbiguanide hydrochloride was administered in water solution by means of a stomach tube to 24 hr. fasted male Sprague-Dawley rats in doses of 100 mg./kg. body weight. The urine of the rats was collected at 8-hr. intervals for a 24-hr. period during which time the rats had access only to water. The urine, which was approximately 25 ml. per rat, was frozen upon collection and subsequently lyophilized.

Ion-Exchange Chromatography—The lyophilized urine was slurried with water, in approximately $^1/_6$ the original volume of urine, and the insoluble material removed by centrifugation. The clear solution was then placed on a 20 × 1.25-cm. column of Amberlite CG-50 (carboxylic acid—H^+ form) ion-exchange resin. This column retained all of the inorganic cations as well as strong organic bases. The column was rinsed with water and the eluate passed over a 20 × 1.25-cm. column of Amberlite CG-120 (sulfonic acid — H^+ form). The CG-120 resin retains all weak organic bases. The eluate from this column contains the neutral and acidic components of the urine. All of the radioactive components of the urine were retained by these columns and a summary of the distribution of this radioactivity is given in Table I.

The two columns were then separately eluted, the CG-50 resin with 0–0.1 *N* hydrochloric acid in a gradient elution manner, and the CG-120 resin with 0–0.5 *N* ammonium hydroxide by similar methods. The eluate from the CG-50 column thus contained the strong organic bases while the eluate from the CG-120 resin contained the weak bases.

The remainder of the administered radioactivity (45%) was found to be present in the stools of the animals. This radioactive material was soluble in water and upon separation on a CG-50 (H^+) column (as above) was found to consist of 77% strong base material. These results are also included in Table I.

Weak Base Metabolite—The isolated weak base fraction from 29 rats was extracted with 70 ml. of hot ethanol, the solution filtered, and the residue separated. Although a small amount of radioactivity was taken up in the extract, the majority of the material in this solution consisted of nonradioactive creatine and creatinine (as determined by chromatographic and spectroscopic means). The residue was then extracted with methanol, and isopropyl alcohol was added to the point of saturation. Concentration of the solution yielded three crops of material which gave a single biguanide-positive spot after paper chromatographic separation. These spots corresponded directly to the radioactive area.

Table I—Percentage Distribution of Excreted Radioactive Metabolites

	Total	Strong Base	Weak Base
Urine	56	28	28
Feces	45	35	Undetermined

In order to further purify the radioactive metabolite, 60 mg. obtained from the first three crops was placed on a column of pressure packed Whatman No. 1 powdered cellulose in *n*-butanol saturated with water. The column was eluted with 1.5 vol. of saturated butanol followed by a saturated butanol–acetic acid (3:1) solution. The fractions were collected in 10-ml. aliquots. The radioactivity came off the column in fractions 21–29. The main fraction was extracted with ether and the aqueous solution was then stripped to dryness to give 29 mg. of purified material.

Acid Hydrolysis of the Weak Base Metabolite—A solution of 3 mg. of the purified metabolite in 0.3 ml. of (1*N*) hydrochloric acid was heated for 2 hr. at 100°. At the end of the heating period 0.06 ml. was tested with barium chloride and the negative result indicated the lack of "ethereal sulfate" in the metabolite. A small portion (0.03 ml.) of the hydrolysate was tested for the presence of reducing sugar by the method described by Nelson (7). This test was strongly positive. A similar portion (0.03 ml.) was tested for the presence of phenol by the Folin-Ciocalteu method (8).

A comparison of the results obtained after hydrolysis with a blank run on the unhydrolyzed compound showed that approximately 1 mole of reducing sugar (using glucuronic acid for a standard curve) was released per mole of phenolic group obtained (using phenol for a standard curve).

Incubation with β-Glucuronidase—Incubation of the purified metabolite (5.2 mg.) with β-glucuronidase (5.7 mg., Sigma Chemical Co.) in 5 ml. of sodium acetate-acetic acid buffer (pH 5.8) was carried out for 18 hr. at 37° (9). After this time the protein was denatured with 1 ml. of 5% trichloroacetic acid, and the residue filtered off. The filtered material was washed with 0.2 ml. of water and the combined filtrates extracted with four 2-ml. portions of ether in order to remove excess trichloroacetic acid. The aqueous layer was aerated and then added to a volumetric flask and diluted to 10 ml. When the hydrolysate was assayed for phenol (8) and reducing sugar (7), the ratio was found to be approximately 1:1 (using the standard curves described above).

Strong Base Metabolite—The strong base fraction from 22 rats (335 mg.) was extracted with methanol. Almost the entire residue dissolved. The material was crystallized by means of concentration of the methanol solution and subsequent addition of isopropyl alcohol. These extracts contained the majority of the radioactivity present. The extracts were evaporated to dryness and the solid taken up in water to be used for chromatography.

Synthesis of p-Hydroxy-β-phenethylbiguanide Hydrochloride—Tyramine hydrochloride (2.5 g., 0.014 mole) was added to cyanoguanidine (1.8 g., 0.014 mole) in a 10-ml. conical flask. The flask

was heated in an oil bath to the fusion point (oil bath 175°) and then the bath temperature was raised to 190° for 2 hr. Upon cooling the mixture solidified. The solid glass-like material was dissolved in methanol and the solution concentrated until a precipitate was obtained upon cooling. The precipitate contained considerable unreacted starting material. One-half of the filtrate was added to a solution of cupric chloride dihydrate in 10 ml. of water and the pH adjusted to 5.6. The precipitated copper biguanide complex, along with any copper hydroxide formed, was centrifuged and washed with two 10-ml. portions of water. The solid material was slurried in 20 ml. of water and hydrogen sulfide gas was passed through the slurry for 1 hr. When the formation of copper sulfide was complete, the material was filtered and the solution lyophilized.

The grayish solid was extracted with methanol and the insoluble copper sulfide residue filtered off. The solution was evaporated to dryness and taken up in a minimum of hot ethanol. Upon cooling, 115 mg. of white crystals were obtained, m.p. 181–186°. Recrystallization from acetonitrile gave white crystals, m.p. 180–183°.[1] The NMR spectrum of this compound in trifluoroacetic acid confirms the structure as *p*-hydroxy-β-phenethylbiguanide. The symmetric aromatic quartet [$\delta = -7.21$ p.p.m.][2] is characteristic of *p*-substituted aromatic rings. The nitrogen protons of the protonated biguanide are observed slightly downfield of the aromatic protons. The methylene protons exhibit peaks at [$\delta = -3.13$ p.p.m.] and [$\delta = -3.92$ p.p.m.].[3]

The ultraviolet spectrum in water shows the absorption ($\lambda_{max.}$ 227 mμ, ε 15.8 × 10³) characteristic of the biguanide conjugated system (11). The infrared spectrum was consistent with the proposed structure [*e.g.*, NH peaks 3,320 cm.$^{-1}$, 1,653 cm.$^{-1}$ (12); aromatic 1,4-disubstitution peaks 1,620 cm.$^{-1}$, 832 cm.$^{-1}$] (13).

Chromatographic Identification of the Metabolite—The hydrolyzed weak base metabolite, the purified strong base metabolite, and synthetic *p*-hydroxy-β-phenethylbiguanide were chromatographed in acidic, neutral, and basic solvent systems. The results of these chromatograms are given in Table II. In all three systems the strong base metabolite, the weak base hydrolysate, and the synthetic *p*-hydroxy-β-phenethylbiguanide show the same R_f values, indicating that the three are identical.

Spectroscopic Identification of the Metabolites—The ultraviolet absorption spectra of synthetic *p*-hydroxy-β-phenethylbiguanide hydrochloride in neutral and basic solution were compared to the spectra obtained with the weak base hydrolysate, and the strong base metabolite in identical solutions. In all cases these spectra were identical, thus confirming that the metabolite was *p*-hydroxy-β-phenethylbiguanide hydrochloride.

The oxidation of aromatic rings in the process of metabolism is a well documented process (1). The observation that β-phenethylbiguanide is hydroxylated *in vivo* may have important implications as to its mode of action. Work on the physiological activity of this metabolite is currently under way.

[1] Analytically pure *p*-hydroxy-β-phenethylbiguanide hydrochloride subsequently supplied by U. S. Vitamin and Pharmaceutical Corp., New York, N. Y., was found to have identical physical characteristics as this material.

[2] Relative to external tetramethylsilane.

[3] The analysis of the structure and NMR spectra of protonated biguanides have been reported elsewhere (10). The readers are referred to the MS thesis of P. J. Murphy for the original spectra.

TABLE II—R_f VALUES OF URINARY METABOLITES AND CONTROLS[a]

Compound	R_f Value in Solvent System[b] A	B	C
Weak base (conj.)	0.25	0.20	—
Weak base (hydrol.)	0.17[c]	0.35[c]	0.65[c]
Strong base	0.17[d]	0.35[d]	0.65[d]
p-Hydroxy-β-phenethylbiguanide	0.17	0.35	0.65
β-Phenethylbiguanide	0.50	0.67	0.74

[a] Ascending technique at room temperature. [b] Solvent systems; A, *n*-butanol, saturated with water; B, *n*-butanol–formic acid–water (63:17:20); C, methanol–pyridine–water (80:2:20). [c] In each system the weak base hydrolysate gave a radioactive spot lower than the main spot. This spot is attributed to incomplete hydrolysis. [d] The reported spot is the major guanidine-positive spot, however a much weaker positive area was observed closer to the origin on all chromatograms. This spot is not presently explainable.

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Keyphrases

β-Phenethylbiguanide (C-14 labeled)—metabolism
Metabolites—isolated, identified
Radioactivity determination—relative, specific
Column chromatography—separation
Paper chromatography—identity
UV spectrophotometry—structure
IR spectrophotometry—structure
NMR spectrometry—identity