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J Clin Invest. 1983;**71**(5):1289-1296. <https://doi.org/10.1172/JCI110879>.

Research Article

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Isolation and Chemical Characterization of 2-Hydroxybenzoylglycine as a Drug Binding Inhibitor in Uremia

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ABSTRACT An organic compound that inhibits drug binding in uremia has been isolated from the sera of chronic renal failure patients, and its chemical structure has been determined. Addition of the compound to normal human sera in vitro resulted in drug binding defects similar to those seen in uremia. The purification of this substance was accomplished by *n*-butyl chloride extraction of acidified (pH 3.0) uremic sera followed by column chromatography, thin-layer chromatography, and paper electrophoresis. From analytical studies including ultraviolet and fluorescence spectroscopy, gas chromatography, chemical ionization and electron impact mass spectrometry, and proton nuclear magnetic resonance spectroscopy, the chemical structure of the uremic binding inhibitor was deduced to be 2-hydroxybenzoylglycine. This confirms the hypothesis that the drug binding defect in uremia is due to the accumulation of endogenous metabolic products rather than an intrinsic structural defect in albumin.

INTRODUCTION

Uremia is associated with a variety of biochemical and metabolic abnormalities that persist despite effective dialysis along with meticulous biochemical monitoring and pharmacological supplementation. These abnormalities, however, usually respond to successful renal transplantation. In uremia, the absorption, protein binding, distribution, and metabolism of drugs is similarly altered. The change in drug binding to serum

protein could potentially affect the therapeutic efficacy or toxicity of various pharmacological agents.

It is widely recognized that in uremic patients' sera, the protein binding of many acidic drugs is reduced while the binding of neutral or basic drugs tends to be unaltered (1-3). There are two major hypotheses to explain these binding defects: (a) there may be intrinsic structural changes in albumin, thus making it abnormal in its drug binding capacity (4, 5) and (b) there may be accumulation of endogenous metabolic products that could tightly bind to albumin, and compete with drug binding (6-10). Recently, evidence has been accumulating to support the second hypothesis. Evidence includes: (a) rapid correction of binding defects by successful renal transplantation (11) and (b) in vitro correction of binding defects by activated charcoal treatment of acidified uremic sera (7) and extraction of acidified sera with *n*-butyl chloride (9) and anion exchange resin (10). This paper reports the isolation and identification of a compound present in uremic sera that can cause binding defects when added to normal human sera in vitro, thus mimicking the binding defects seen in uremia.

METHODS

Chemicals. Chemicals were of the highest purity available. 5,5-Diphenyl [4-¹⁴C]hydantoin and 3- α -acetyl[α -¹⁴C]benzyl-4-hydroxycoumarin were purchased from Amer sham Corp., Arlington Heights, IL; and [G-³H]digitoxin, [7-¹⁴C]2-hydroxybenzoic acid, and [methyl-³H]diazepam were purchased from New England Nuclear, Boston, MA. Nafcillin was supplied by Wyeth Laboratories, Div. American Home Products Corp., Philadelphia, PA; sulfamethoxazole, trimethoprim, and diazepam were supplied by Hoffman-LaRoche, Inc., Nutley, NJ.

Serum sample. Uremic serum samples were obtained with informed consent from patients with chronic renal failure maintained on hemodialysis; the specimens were col-

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Received for publication 30 August 1982 and in revised form 23 December 1982.

lected immediately before hemodialysis and before administration of heparin. Normal healthy volunteers were used to obtain either pooled or individual serum specimens to be used as controls. Serum specimens were kept frozen at -20°C until use.

Protein binding measurements. Equilibrium dialysis as described previously (9) was used to determine the degree of protein binding. Drug binding values obtained by this method were used to estimate the normal range of protein binding, the extent of drug binding defects in uremia, and the degree of binding defects induced in normal human sera by addition of 2-hydroxybenzoylglycine. Final concentrations of the drugs used in protein binding studies were: sulfamethoxazole, 20 $\mu\text{g}/\text{ml}$; nafcillin, 20 $\mu\text{g}/\text{ml}$; trimethoprim, 20 $\mu\text{g}/\text{ml}$; 5,5-diphenylhydantoin, 5 $\mu\text{g}/\text{ml}$; digitoxin, 2 ng/ml ; 3- α -acetylbenzyl-4-hydroxycoumarin, 2 $\mu\text{g}/\text{ml}$; 2-hydroxybenzoate, 100 $\mu\text{g}/\text{ml}$; and diazepam, 2 $\mu\text{g}/\text{ml}$.

Preparation of the extract with drug binding inhibitor from uremic serum. Normal and uremic sera were extracted with *n*-butyl chloride according to the method previously described (9). The organic solvent extract prepared from a 5-ml serum sample was evaporated to dryness in vacuo and dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4), the pH adjusted to 3.0, and the sample was applied to a 0.1 N HCl-washed XAD-2 column. The column was washed with deionized water, and methyl alcohol was then used to elute the material from the column. The extract was then taken to near dryness in vacuo.

Thin-layer chromatography (TLC)¹ extracts. Aliquots of normal or uremic serum extracts were applied to silica gel 60 plates (E. Merck, Darmstadt, West Germany) and developed by ascending chromatography in petroleum ether/ethyl alcohol (absolute)/glacial acetic acid (24:6:7:2 [vol/vol/vol/vol]). Ultraviolet light (254 and 365 nm) was used to visualize any light-absorbing or fluorescing compounds present in the extract. Areas of the chromatogram with fluorescent bands as well as areas between those bands were scraped from the plate, and the material of interest was eluted from the silica gel with methyl alcohol and then filtered through a sintered glass funnel to remove the silica. The filtrate was evaporated to near dryness in vacuo.

Assessment of the number of compounds in the extract capable of inducing binding defects in vitro. Individual fractions of the extracts on thin-layer chromatogram were scraped and eluted as described in the previous section. The ability of one or more fraction to induce binding defects for 5,5-diphenylhydantoin or sulfamethoxazole in normal human sera in vitro was determined by protein binding measurements as described previously.

Correlation of degree of binding defects with amount of uremic binding inhibitor. 5-ml samples of pooled normal sera and 12 uremic serum specimens were collected. 2 ml of each specimen was used to determine the binding values for sulfamethoxazole as described above and 3.0 ml was used to prepare extracts as described above. The extracts were applied to a silica gel TLC plate and developed in the system described above. For each normal or uremic extract, an area with an R_f value of 0.43 was scraped from the plate and the material eluted with methyl alcohol. Each sample was excited at 305 nm and the fluorescent emission at 425 nm was measured.

Purification of material for gas chromatography-mass

spectroscopy. The biologically active dried residue with an R_f value of 0.43 on TLC was dissolved in 0.5 ml of methyl alcohol and applied to a sheet of Whatman 1-mm chromatography paper (Whatman Laboratory Products Inc., Whatman Paper Div., Clifton, NJ). Low voltage electrophoresis was performed by using a Gelman Macro Electrophoresis Chamber (Gelman Sciences, Inc., Ann Arbor, MI) and 0.1 M ammonium carbonate (pH 8.9). Voltage (250 voltage direct current; 18 mA) was applied at room temperature for 1.5 h. The suspect compound migrated 6 cm from the point of application towards the anode. The paper was air dried and the compound was eluted from the paper with 15 ml of methyl alcohol. The methyl alcohol was removed by evaporation.

Direct inlet chemical ionization mass spectroscopy. The molecular weight of the uremic binding inhibitor was determined by direct inlet chemical ionization mass spectroscopy. A Du Pont 102 gas chromatograph-mass spectrometer (Du Pont Co., Analytical Instruments Div., Wilmington, DE) with Hewlett-Packard 2113B data system (Hewlett-Packard Co., Palo Alto, CA) equipped with a chemical ionization source was used with isobutane as reagent gas. The instrument was calibrated with perfluorotributylamine. The ion source was set to a temperature of 220°C . Mass spectra were obtained at an electron energy of 75 eV, an emission current of 500 μA , and scanning from 75 to 500 amu (atomic mass units) at 125 amu/s. Samples were introduced through a direct probe.

Trimethylsilylation and electron impact mass spectroscopy. Trimethylsilyl derivatives were prepared by adding 20 μl of *N*-O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Regis Chemical Co., Morton Grove, IL) to 10 μg of uremic binding inhibitor or 50 μg of each of the isomers of hydroxybenzoylglycine. The vials were capped and heated at 75°C for 0.5 h. Just before chromatography, the excess derivatizing reagent was evaporated under a stream of dry nitrogen. Samples were reconstituted in dry dichloromethane that was stored over sodium sulfate. Mass spectra were acquired after the gas chromatographic introduction of 2 μl of the reaction mixture into the Du Pont 102 gas chromatograph-mass spectrometer under the following conditions: 5 ft \times 2 mm i.d. 3% Dexsil 400 (Supelco, Inc., Bellefonte, PA) packed column; helium, 27–28 ml/min; oven temperature at 240°C ; injector temperature at 250°C ; 75 eV ionizing energy; the ion source and separator temperature at 220 and 250°C , respectively; masses were scanned from 25 to 500 amu at 125 amu/s.

Chemical synthesis of hydroxybenzoylglycine isomers. A modification of the active ester method (12) was used to synthesize 2-, 3-, and 4-hydroxybenzoylglycines. In the synthesis of the *N*-hydroxysuccinimide esters of the hydroxybenzoic acids, half the recommended amount of dioxane was used. The *N*-hydroxysuccinimide esters were not isolated but were immediately converted into the corresponding glycine derivatives. Before purification of the hydroxybenzoylglycines, only half of the dioxane was removed before extracting the reaction mixture with ethyl acetate. After discarding the ethyl acetate layer, the aqueous layer was acidified to pH 2 and stored at 4°C overnight to insure maximum precipitation of the hydroxybenzoylglycine. After recrystallization from water, melting points of the dried crystals were taken by using a Thomas "Uni-Melt" Capillary Melting Point Apparatus (Arthur H. Thomas Co., Philadelphia, PA) at a $1^{\circ}\text{C}/\text{min}$ increase in temperature. Ultraviolet scans of the pure compounds in methyl alcohol were obtained by using a Perkin-Elmer (Coleman 124; Perkin-Elmer Corp. Instrument Div., Norwalk, CT) Scanning Double Beam Spectrophotom-

¹ Abbreviations used in this paper are: amu, atomic mass unit; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

eter. Fluorescence emission spectra of the pure compounds were obtained after excitation of a 3-ml sample in methyl alcohol at 305 nm by using an Aminco-Bowman model J4-8962 spectrofluorometer (SLM Instruments, Inc., Urbana, IL).

Nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy was performed by using a JEOLCO FXQ90 NMR spectrometer operating at 89.55 MHz in the Fourier transform mode. Methyl alcohol- d_4 was used as the solvent and 190 transients were collected as a pulse.

Effect of 2-hydroxybenzoylglycine on protein binding in normal human serum. To study the effect of increasing concentrations of 2-hydroxybenzoylglycine on protein binding in normal human serum in vitro, 2-hydroxybenzoylglycine was added to pooled normal human serum to give final concentrations of 0, 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mM; samples were dialyzed against 20 vol of Krebs-Ringer phosphate buffer (pH 7.4) for 48 h before protein binding value determination. Protein binding values for 5,5-diphenylhydantoin, digitoxin, 3- α -acetylbenzyl-4-hydroxycoumarin, 2-hydroxybenzoate, diazepam, and trimethoprim at each concentration of 2-hydroxybenzoylglycine were determined as described previously (9).

RESULTS

Isolation of a uremic binding inhibitor. Extraction of acidified uremic serum with *n*-butyl chloride revealed several fluorescent bands that were not present

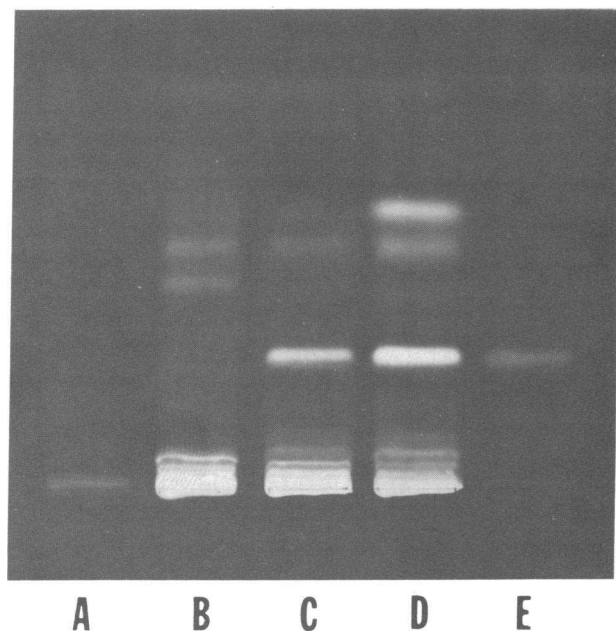


FIGURE 1 Thin-layer chromatogram of serum extracts. Lane A, extract of normal human serum; lanes B, C, and D, extracts of three different uremic sera; lane E, uremic binding inhibitor just before final purification by electrophoresis. Solvent system was petroleum ether/ether/absolute ethyl alcohol/glacial acetic acid (24:6:7:2 [vol/vol/vol/vol]) and detection was accomplished by visualization with shortwave (254 nm) ultraviolet light.

in the extracts of normal human serum. Fig. 1 is a representative example of normal and uremic serum extracts chromatographed on silica gel 60 TLC plates with a solvent system of petroleum ether/ether/ethyl alcohol/glacial acetic acid (24:6:7:2 [vol/vol/vol/vol]). Compounds were located by exposing the TLC plate to short wave (254 nm) ultraviolet light. An extract of normal human serum and extracts of three different uremic sera are shown in lanes A–D, respectively. Lane E shows the compound with an R_f value of 0.43 isolated from uremic extracts that could cause binding defects when added to normal human serum in vitro. The compound shown in lane E was further purified by paper electrophoresis for the following structural analysis.

Correlation of degree of binding defects observed with the amount of uremic binding inhibitor present. The amount of fluorescent material with an R_f value of 0.43 on TLC plates was compared with the severity of binding defects seen in their corresponding samples. As can be seen in Fig. 2, there is a good correlation between the relative intensity of fluorescence at 425 nm present in the material isolated from uremic serum and the severity of binding defects for sulfamethoxazole. Similar results were obtained when the relative intensity of fluorescence at 425 nm was compared with the binding values for nafcillin in the corresponding serum samples from which the extracts were obtained (data not shown).

Chemical synthesis of hydroxybenzoylglycine isomers. 2-, 3-, and 4-hydroxybenzoylglycine were synthesized as described in Methods. Yields were 80, 47, and 50% for 2-, 3-, and 4-hydroxybenzoylglycine, respectively. Melting points and ultraviolet absorption scans of the synthesized compounds gave excellent agreement with those reported in the literature as shown in Table I.

Direct inlet chemical ionization mass spectrometry. The mass spectrum of the uremic binding inhibitor gave a definitive peak at m/z (ratio of mass to charge) 196 corresponding to its protonated molecular ion and allowed the determination of the compound's molecular weight.

Electron impact mass spectrometry. Electron impact mass spectrometry was performed to determine which isomer of hydroxybenzoylglycine the uremic binding inhibitor might be. Bis-trimethylsilyl-derivatives of 2-, and 3-, and 4-hydroxybenzoylglycine and the uremic binding inhibitor were prepared as described in Methods and were used to obtain the mass spectra shown in Fig. 3. Panel A in Fig. 3 shows the electron impact mass spectrum of the bis-trimethylsilyl-derivative of the uremic binding inhibitor, and panel B in Fig. 3 shows the electron impact mass spectra of the bis-trimethylsilyl-derivative of 2-hydroxy-

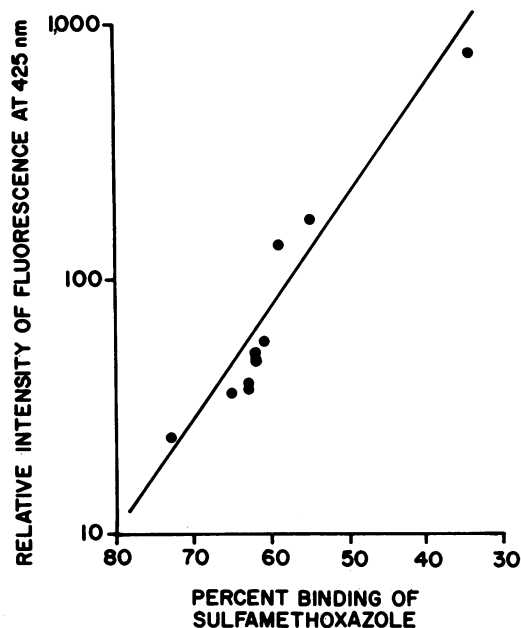


FIGURE 2 Correlation of degree of binding defects observed with the amount of uremic binding inhibitor present. 5-ml samples of pooled normal human sera and 12 individual uremic serum specimens were collected. 2 ml of each specimen were used to determine the protein binding values for sulfamethoxazole (described in Methods) and 3.0 ml was used to prepare extracts as described in Methods. Extracts were applied to silica gel 60 plates and developed in the solvent system elaborated in Fig. 1. For each extract, an area of the TLC plate with an R_f value of 0.43 was scraped from the plate and the material eluted with methyl alcohol. Each sample was excited at 305 nm and the fluorescent emission at 425 nm was measured. The relative intensity of fluorescence at 425 nm (graphed on the Y-axis) was plotted against the protein binding value for sulfamethoxazole (graphed on the X-axis) from the corresponding serum sample from which the extract was obtained.

benzoylglycine. All compounds show a molecular ion at m/z 339 that would be typical of a hydroxybenzoylglycine substituted with two trimethylsilyl groups (one on the carboxyl-terminal hydroxyl group and the other on the phenolic hydroxyl group). The bis-trimethylsilyl derivative of the uremic binding inhibitor (panel A, Fig. 3) shows prominent peaks at m/z 206 and 324 that are also present in the same proportion in the mass spectrum of the bis-trimethylsilyl derivative of 2-hydroxybenzoylglycine (panel B, Fig. 3), but are not nearly as prominent in the mass spectra of the bis-trimethylsilyl-derivatives of 3- and 4-hydroxybenzoylglycine. Bis-trimethylsilyl derivatives of 3- and 4-hydroxybenzoylglycine have very large peaks at m/z 294. The peak at m/z 294 is not prominent in either the mass spectrum of the bis-trimethylsilyl derivatives of the uremic binding inhibitor (Fig. 3, panel A) or 2-hydroxybenzoylglycine (Fig. 3, panel B). Other dif-

ferences between the mass spectra can be seen. Both the bis-trimethylsilyl derivatives of 2-hydroxybenzoylglycine and the uremic binding inhibitor have a small peak at m/z 267 that is not noticeably present in the bis-trimethylsilyl derivatives of 3- or 4-hydroxybenzoylglycine. The computerized data system with >35,000 mass spectra on file identified the uremic binding inhibitor as 2-hydroxybenzoylglycine on the basis of comparison to library spectra.

Proton NMR spectroscopy. Proton NMR studies were undertaken to establish the exact position of the phenolic hydroxyl group in the molecule. The chemical shifts and spin-coupling patterns of the four aromatic protons (Fig. 4) confirmed the proposed structure as 2-hydroxybenzoylglycine. In particular, the lowest field proton (H_d) showed splittings of 8.5 and 1.5 Hz, establishing the presence of one *ortho* and one *meta* coupling. This is consistent only with formulation as the *ortho* isomer. The glycine methylene was represented by a two-proton singlet at δ 4.13.

Other data confirming the structure of the uremic binding inhibitor. Other tests confirmed the structure of the uremic binding inhibitor as 2-hydroxybenzoylglycine. Table I shows a comparison of the ultraviolet absorption, fluorescence, and chromatographic properties of the uremic binding inhibitor compared with those of authentic 2-, 3-, and 4-hydroxybenzoylglycine. As can be seen, the ultraviolet absorption, fluorescence emission maxima, and chromatographic behavior of 2-hydroxybenzoylglycine and the uremic binding inhibitor are identical to each other, while noticeably different from 3- and 4-hydroxybenzoylglycine. The chromatographic behavior of the uremic binding inhibitor and 2-hydroxybenzoylglycine are also similar to one another (R_f in TLC of 0.33 and 0.33; retention time in gas chromatography of 1.55 and 1.57 min) while different from 3- and 4-hydroxybenzoylglycine (R_f value of 0.15 and 0.14, respectively; retention time in gas chromatography of 1.92 and 2.60 min, respectively).

Effect of adding known amounts of 2-hydroxybenzoylglycine on protein binding in normal human serum in vitro. To study the effects of 2-hydroxybenzoylglycine on serum protein binding in vitro, increasing amounts of 2-hydroxybenzoylglycine were added to serum and protein binding values for several drugs were obtained. Fig. 5 shows the effects of 2-hydroxybenzoylglycine on the protein binding of 5,5-diphenylhydantoin (panel A) and trimethoprim (panel B). As can be seen, 2-hydroxybenzoylglycine inhibits the protein binding of 5,5-diphenylhydantoin (panel A), but does not affect the protein binding of trimethoprim (panel B). As can also be seen in Fig. 5, panel A, increasing the concentration of 2-hydroxybenzoylglycine causes a greater decrease in the protein bind-

TABLE I
Properties of Hydroxybenzoylglycine Isomers and the Uremic Binding Inhibitor

Property	4-Hydroxybenzoylglycine	3-Hydroxybenzoylglycine	2-Hydroxybenzoylglycine	Uremic binding inhibitor
Melting point in °C*	239–240 (240)	190–191 (191)	167–168 (168)	—
UV absorption maximum in nanometers	254 (254)	292 (292)	237, 301 (237, 301)	237, 301
Fluorescence emission maximum in nanometers§	360, shoulder 320	355	425, shoulder 350	425, shoulder 350
R_f value	0.14 (0.14)	0.15 (0.15)	0.33 (0.33)	0.33
Retention time in minutes¶	2.60	1.92	1.57	1.55

* See Methods for experimental details. Literature values (12) are given in parentheses.

† See Methods for experimental conditions. Compounds were dissolved in methyl alcohol. Literature values (12) are given in parentheses.

§ Compounds were dissolved in methyl alcohol and excited at 305 nm. Fluorescence emission spectra were determined as described in Methods.

^{||} R_f values are for compounds on silica gel 60 plates using the following solvent system: toluene/ethyl formate/formic acid (5:4:1 [vol/vol/vol]). Literature values (13) are given in parentheses.

¶ Retention times are given for the bis-trimethylsilyl-derivatives of compounds on a packed Dexsil 400 column. Gas chromatographic conditions are given in Methods.

ing of 5,5-diphenylhydantoin. Increasing the concentration of 2-hydroxybenzoylglycine also causes a decrease in the protein binding of digitoxin, 3- α -acetylbenzyl-4-hydroxycoumarin, 2-hydroxybenzoate, and diazepam (data not shown). The effect of 2-hydroxybenzoylglycine on serum protein binding is much like that observed in uremia. In uremia, the binding of 5,5-diphenylhydantoin, digitoxin, 3- α -acetylbenzyl-4-hydroxycoumarin, 2-hydroxybenzoate, and diazepam is decreased, while the binding of trimethoprim is normal.

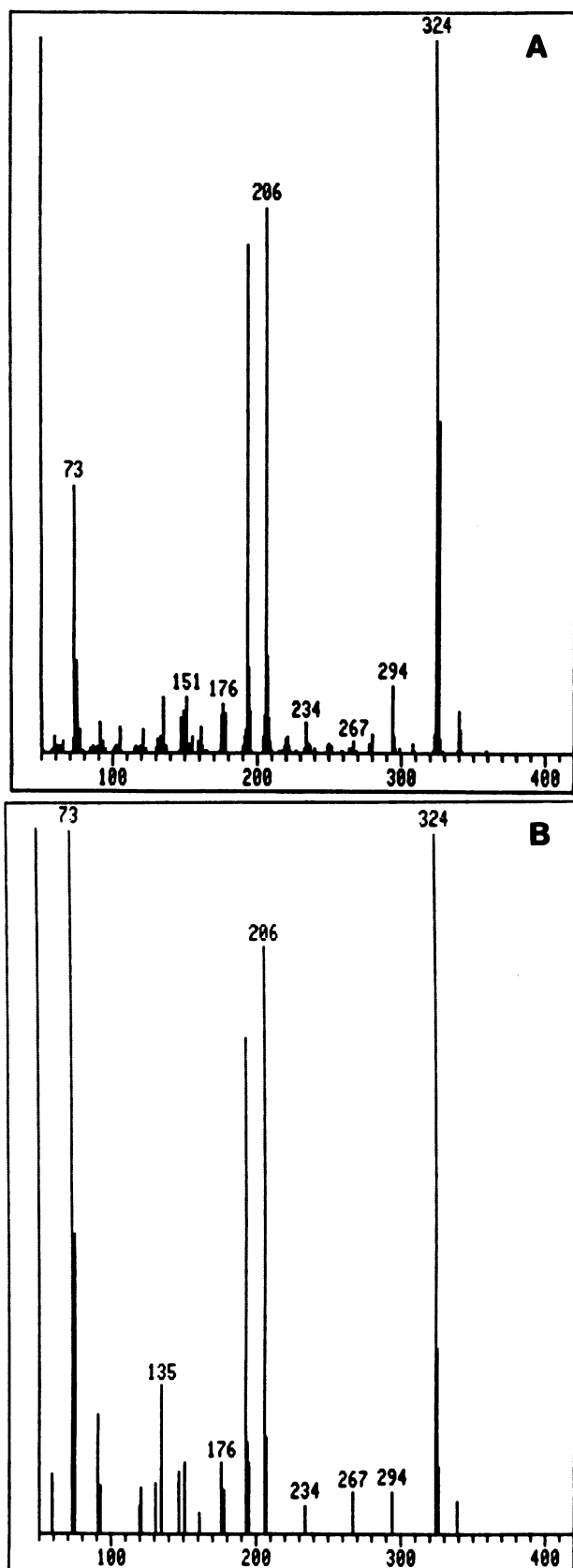
DISCUSSION

Uremia is a relatively poorly defined syndrome resulting from a multisystem failure secondary to severe renal failure. It is believed that the syndrome is a result of the toxic effects of certain substances that are normally excreted by the kidneys, but accumulate in renal failure. Many endogenous compounds are known to accumulate in the sera of patients with moderate to severe renal failure. However, no specific quantitative correlation has been made between the levels of certain compounds and given uremic symptoms.

Defective drug binding in uremia is a well established phenomenon, although its precise mechanism to explain the findings is unknown. We have previously demonstrated that the binding inhibitor could readily be recovered from either an *n*-butyl chloride extraction of acidified uremic serum (9) or anion exchange resin exposed to uremic serum (10). The compound was shown to be acidic in nature with a mol wt < 500. It was therefore speculated that the compound most

likely represents one of the endogenous metabolic products tightly bound to albumin at physiologic pH but extractable at acidic pH.

The present study reports the chemical structure of this compound. The chemical ionization mass spectrum of the compound conclusively established the molecular weight of the uremic binding inhibitor to be 195. This is in agreement with our previous findings indicating the mol wt to be <500. (9). Electron impact mass spectrometry revealed very similar mass fragmentation patterns between the uremic binding inhibitor and 2-hydroxybenzoylglycine. The mass fragmentation patterns of 3- and 4-hydroxybenzoylglycine were strikingly different than those of either 2-hydroxybenzoylglycine or the uremic binding inhibitor. Proton NMR studies conclusively established that the phenolic hydroxyl group was *ortho*, thus establishing the structure as 2-hydroxybenzoylglycine. Other analytical evidence supported the chemical identity of the uremic binding inhibitor to be 2-hydroxybenzoylglycine. This evidence included similar retention times in gas chromatography, identical R_f in several TLC systems, identical ultraviolet absorption and fluorescence emission spectra, and identical migration on paper electrophoresis. Protein binding studies with pure 2-hydroxybenzoylglycine revealed similar properties of this compound with the binding defects seen in uremia. 2-Hydroxybenzoylglycine was able to induce binding defects for 5,5-diphenylhydantoin, digitoxin, 3- α -acetylbenzyl-4-hydroxycoumarin, 2-hydroxybenzoate, and diazepam. In uremia, the binding of these five drugs is also inhibited. The binding of trimethoprim was unaltered by 2-hydroxybenzoylgly-



cine; the binding of trimethoprim is normal in uremic patients.

A preliminary study designed to examine the effect of varying levels of the inhibitor on the extent of binding defects demonstrated a strong positive correlation between the two parameters. The levels of binding inhibitor were approximated by the fluorescent intensity. Studies are in progress to develop a more precise quantitative method to accurately measure the levels of 2-hydroxybenzoylglycine present in individual uremic serum specimens.

Future studies will be undertaken to determine the metabolic pathway involved in the synthesis of 2-hydroxybenzoylglycine. In renal failure, protein catabolism is greatly increased. The concentrations of many amino acids (both essential and nonessential) are decreased in uremia (14). Tryptophan is an essential amino acid whose serum concentration and whose binding to albumin is decreased in uremia (15). Tryptophan metabolites have been reported to be increased in uremic serum (16). Products of tryptophan catabolism appearing in normal human urine are indole-3-acetic acid, 2-aminobenzoic acid, α -2-diamino- γ -oxo-benzenebutanoic acid, 4-hydroxy-2-quinolinecarboxylic acid, and 4,8-dihydroxy-2-quinolinecarboxylic acid (17). 2-Hydroxybenzoic acid could arise from 2-aminobenzoic acid. Phenolic acids such as 2-hydroxybenzoic acid could be conjugated with glycine in the liver to give 2-hydroxybenzoylglycine. Glycine conjugates of 2-, 3-, and 4-hydroxybenzoic acids are all found in normal human urine (18).

2-Hydroxybenzoylglycine has been isolated from the urine of healthy, aspirin-free individuals who had ingested an oral dose of L-tryptophan. 2-Hydroxybenzoic acid was thought to arise from L-tryptophan by bacterial action in the colon, since 2-hydroxybenzoylglycine could not be found in the urine of patients who were given neomycin to inhibit bacterial growth (19). Compared to oral doses of L-tryptophan, direct introduction of this amino acid into the colon resulted in a greater production of 2-hydroxybenzoylglycine.

Exogenous sources of 2-hydroxybenzoylglycine are multiple. They include some obvious sources such as aspirin or other salicylate-containing medications, while some sources are not as obvious. Hydroxybenzoates are known to be naturally present in many fruits

FIGURE 3 Electron impact mass spectra of bis-trimethylsilyl-derivatives of the uremic binding inhibitor and 2-hydroxybenzoylglycine. Panel A, bis-trimethylsilyl-derivative of the uremic binding inhibitor; panel B, bis-trimethylsilyl-derivative of 2-hydroxybenzoylglycine. Bis-trimethylsilyl-derivatives were prepared as described in Methods. Mass spectra were acquired after the gas chromatographic introduction of the sample into a Du Pont 102 gas chromatograph-mass spectrometer with a Hewlett-Packard 2113B data system under the conditions described in Methods.

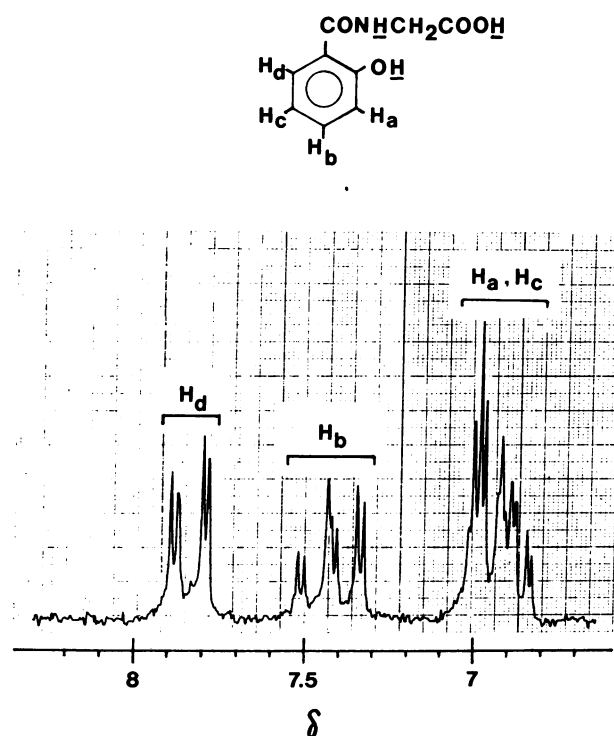


FIGURE 4 Proton NMR Spectrum of the four aromatic protons in the uremic binding inhibitor. NMR spectroscopy was performed by using a JEOLCO FXQ90 NMR spectrometer operating at 89.55 MHz in the Fourier transform mode. Methyl alcohol- d_4 was used as the solvent and 190 transients were collected as a pulse. The chemical shifts and spin coupling patterns of the four aromatic protons (H_a , H_b , H_c , and H_d) are shown.

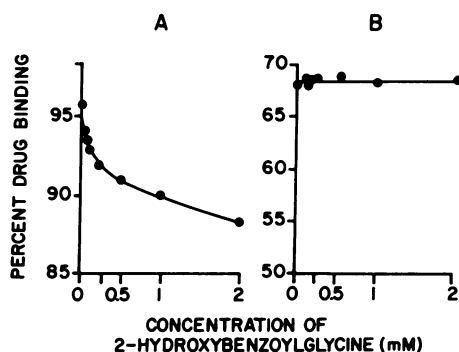


FIGURE 5 Effect of increasing concentrations of 2-hydroxybenzoylglycine on protein binding in vitro. 2-Hydroxybenzoylglycine was added to pooled normal human sera in vitro to give final concentrations of 0, 0.0625, 0.125, 0.25, 0.50, 1.0, and 2.0 mM. Protein binding values for 5,5-diphenylhydantoin and trimethoprim were determined. Panel A, effect of 2-hydroxybenzoylglycine on 5,5-diphenylhydantoin; panel B, effect of 2-hydroxybenzoylglycine on trimethoprim binding.

and vegetables (20), cow's milk (21), food additives, and pharmaceutical products that use wintergreen (methyl salicylate) as flavoring agents (22). Once absorbed, 2-hydroxybenzoic acid could then be conjugated with glycine to form 2-hydroxybenzoylglycine.

Generally, glycine conjugates are considered to be products of detoxification and therefore free of toxic activity. However, detoxification studies are usually made in animals with normal kidneys that are capable of rapidly excreting the detoxified compounds. In the uremic patient, such compounds are retained and circulate through various organs. These retained metabolites may contribute to the symptoms seen in uremia. Some aromatic acids and their derivatives have been shown to inhibit cerebral enzymes (23).

In summary, 2-hydroxybenzoylglycine has been isolated from the sera of uremic patients and shown to be responsible for the drug binding defects seen in uremia. Although this compound may not be the only substance that contributes to drug binding defects present in uremia, it certainly appears that it represents a major metabolic product to cause the defect. Further studies are under progress to elucidate more precise biochemical mechanisms involved in the genesis of the inhibitor and to investigate other roles that it may play in uremia as one of "uremic toxins".

ACKNOWLEDGMENTS

Gas chromatography-mass spectrometry was performed at National Medical Services, Willow Grove, PA. We wish to thank Dr. Fredric Rieders for making the mass spectrometer available to us. We wish to thank Dr. R. John Stedman of the Department of Medicinal Chemistry, Temple University School of Pharmacy for performing the proton NMR studies and interpreting the results. The authors are also indebted to Dr. Michael R. Rudnick and staff members of the Hemodialysis Unit at Temple University Hospital for their assistance in organizing the collection of uremic blood specimens. We are grateful to Dr. Robert Suhadolnik and Dr. Gerhard Plaut in the Department of Biochemistry at the Temple University Health Sciences Center for many useful discussions and comments. The expert secretarial assistance of Bernice Croom is gratefully acknowledged.

This work was supported by grant 5R01AM26807 from the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases.

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