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Influence of Blood Proteins on Biomedical Analysis. IX.[1] Protective Effects of Human Serum Proteins on Anion-Induced Degradation of Gliclazide

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Gliclazide, 1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(*p*-tolylsulfonyl)urea, an oral hypoglycemic drug having the sulfonylurea structure, was completely degraded in a medium containing more than 0.2 M chloride or bromide ion at 37 °C after 24 h. Other sulfonylureas, tolazamide and glycopyramide, were also degraded in the presence of chloride ion, but tolbutamide, acetohexamide, chlorpropamide and glibenclamide were not. Fluoride, carbonate, acetate, sulfate and phosphate ions and both sodium and potassium cations induced slight degradation of gliclazide. Two degradation products were isolated, crystallized, and identified as a hydrazine compound (3-azabicyclo[3.3.0]oct-3-yl-amine monohydrochloride) and *p*-toluenesulfonamide based on mp, ultraviolet, infrared, proton nuclear magnetic resonance, mass spectra and high performance liquid chromatography data. The anion-induced gliclazide degradation was dose-dependently depressed by human serum albumin or other proteins. It was completely depressed by albumin at over 0.3 mg/ml. Other serum proteins, α, β and γ-globulins, depressed the anion (Cl^-)-induced gliclazide degradation similarly to albumin.

Keywords——gliclazide; sulfonylurea; anion-induced gliclazide degradation; hypoglycemic drug; human serum albumin; gliclazide-serum protein binding

Gliclazide (**1**) (1-(3-azabicyclo[3.3.0]oct-3-yl)-3-(*p*-tolylsulfonyl)urea is a "second generation" oral potential hypoglycemic drug having the sulfonylurea structure.[2] Sulfonylureas are metabolized by cytochrome P-450, localized in liver microsomes,[3] and it has been accepted that albumin-bound drugs are not metabolized.[4] However, little is known about the metabolism or degradation of the sulfonylureas in extracellular body fluids such as blood. Recently, we observed that gliclazide was easily degraded in albumin-deficient serum,[1,5] and this degradation was significantly stimulated by chloride ion.[6] This finding is interesting from the viewpoint of drug stability in body fluids and could be important in relation to the drug pharmacokinetics in patients with hypoproteinemia.

In the present study, the degradation of gliclazide in the presence of several anions, halide, acetate, carbonate, sulfate and phosphate, was examined. Moreover, the protective effects of human serum albumin (HSA) and other serum proteins on the anion-induced gliclazide degradation were studied. The degradations of six other sulfonylureas, tolbutamide, chlorpropamide, acetohexamide, glibenclamide, glycopyramide and tolazamide, were also compared.

Experimental

Apparatus——All melting points were measured on a micro melting point hot stage apparatus (Yanagimoto Co., Japan) and are uncorrected. The infrared (IR) and ultraviolet (UV) spectra were recorded on a Hitachi EPI-G3

spectrometer (Hitachi Ltd., Japan) and a Hitachi 220 spectrophotometer (Hitachi Ltd., Japan), respectively. The proton nuclear magnetic resonance (^{1}H-NMR) and mass (MS) spectra were recorded on a JNM-FX 270 spectrophotometer (JEOL, Tokyo, Japan) and a JMS-DX 300 mass spectrometer equipped with a FAB gun (JEOL, Tokyo, Japan). Sodium and potassium ion concentrations were measured on a Hitachi 775 flame photometer (Hitachi Ltd., Japan). A high-pressure liquid chromatograph (HPLC) (TWINCLE, Japan Spectroscopic Co., Tokyo, Japan) equipped with a variable-wavelength spectrometric detector (UVIDEC-100 II, Japan Spectroscopic Co., Tokyo, Japan) and a packed column (i.d. 4.6 × 250 mm) of CDR-10 (a macroporous anion-exchange resin, particle size, 7 μm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan) was used for HPLC analysis.

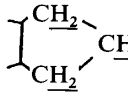
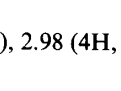
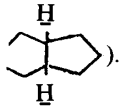
Chemicals——Pure reference samples of gliclazide (Dainippon Pharmaceutical Industries Co., Ltd., Japan), tolbutamide (Hoechst Japan Ltd., Japan), tolazamide (Japan Upjohn Ltd., Japan), glycopyramide (Kyorin Pharmaceutical Co., Ltd., Japan), chlorpropamide (Pfaizer Taito Co., Ltd., Japan), acetohexamide (Shionogi & Co., Ltd., Japan) and glibenclamide (Hoechst Japan Ltd., Japan) were used as received. α-Globulin (human, fraction IV-4, United States Biochemical Co., U.S.A.), β-globulin (human, fraction III, ICN Pharmaceutical Inc., U.S.A.), γ-globulin (human, fraction II, Sigma Chemical Co., U.S.A.) and human and bovine serum albumins (fraction V, Armour Laboratories Ltd., U.S.A.) were used without further purification. A pure reference sample of 3-azabicyclo[3.3.0]oct-3-yl-amine monohydrochloride (**2**) was a gift from the Research Laboratories, Dainippon Pharmaceutical Industries Co., Ltd., Japan. *p*-Toluenesulfonamide (**3**) and all other chemicals were of analytical reagent grade from Wako Pure Chemical Industries Ltd., Japan.

Measurements——i) HPLC Sample: A mixture of 0.5 ml of sample and 1 ml of 0.1 M phosphate buffer (pH 6.9) was shaken vigorously for 15 min with 4 ml of chloroform. The mixture was centrifuged for 15 min at 1400 × ***g***, then 3 ml of the chloroform layer was withdrawn, mixed with 2 ml of 1 N sodium hydroxide, and shaken for 10 min. After centrifugation of the mixture for 5 min, 1.5 ml of the aqueous layer was added to a mixture of 0.5 ml of 3 N hydrochloric acid and 2.5 ml each of 0.1 M phosphate buffer and chloroform, followed by shaking for 10 min. Afrer centrifugation for 5 min, 2 ml of the chloroform layer was evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 0.1 ml of methanol containing the internal standard (methylbenzoate, 8 μg/ml), and a 10-μl aliquot was injected onto the HPLC column.

ii) HPLC: Gliclazide and other sulfonylurea concentrations were determined by HPLC,[7] under the following conditions: column, Diaion CDR-10 (i.d. 4.6 × 250 mm); mobile phase, MeCN–MeOH–1.2 M NH_4ClO_4 (4 : 3 : 7, v/v/v); flow rate, 0.5 ml/min; detector, UVIDEC-100 II (at 227 nm). The measurement was performed at room temperature (25—27 °C), with methylbenzoate as the internal standard. Retention times (min) were: gliclazide 10.75; tolazamide 9.75; glycopyramide 8.75; chlorpropamide 10.2; acetohexamide 9.2; glibenclamide 14.0 (detected at 205 nm; flow rate, 0.7 ml/min); *p*-toluenesufonamide 8.5; internal standard 14.25.

iii) Protein, Sodium and Potassium Ion Concentrations: The protein content of the medium was determined by Lowry's method[8] using bovine serum albumin as the standard. Sodium and potassium ion concentrations were determined by flame photometry.

Degradation of Gliclazide——Gliclazide degradation in medium containing various concentrations of anions was performed as follows: a mixture of 0.05 ml of gliclazide solution (100 μg/ml in 0.067 M phosphate buffer, pH 7.4) and 0.45 ml of the medium containing various concentrations of anion (pH 7.4) was incubated at 37 °C for 24 h, then the concentration of intact gliclazide was determined. The other sulfonylureas were incubated by the same procedure. The degradation rate (%) was calculated as follows: degradation rate (%) = D_{ap}(%) − D_{cont}(%), where D_{ap} and D_{cont} represent the apparent degradation rates in the medium (0.067 M phosphate buffer, pH 7.4) with and without anion or serum protein, respectively.

Isolation and Identification of Degradation Products——Gliclazide (1.5 g) was suspended in 50 ml of 1 N hydrochloric acid containing 1 M sodium chloride. The suspension was refluxed in an oil bath at 100—120 °C for 8 h. The reaction mixture was shaken with 50 ml of chloroform twice. The aqueous layer was adjusted to pH 12 with 2 N sodium hydroxide, then shaken with 50 ml of chloroform twice. The chloroform layers were combined. After evaporation of the chloroform layer to dryness, 1 ml of 1 N hydrochloric acid was added, and the mixture was again evaporated to dryness. Recrystallization of the residue from chloroform–hexane (10 : 1) gave 0.31 g of **2** (46%), mp 180 °C. IR ($CHCl_3$): 2880 (ν_s –CH_2–), 2950 (ν_{as} –CH_2–), 3140 (–NH_3^+), 3250 cm^{-1} (–NH_3^+). ^{1}H-NMR ($CDCl_3$) δ: 1.65—1.73 (6H, br, ), 2.98 (4H, s, –N ), 3.60—3.80 ppm (2H, br, –N ). MS *m/z*: 126 (M^+), 110 ($M^+ - NH_2$). The first chloroform layers were also combined. After evaporation of the chloroform layer to dryness, recrystallization of the residue from chloroform–hexane (10 : 1) gave 0.4 g of **3** (56%), mp 138 °C. UV $\lambda_{max}^{methanol}$ nm: 220, 224. IR ($CHCl_3$): 1160 (ν_s SO_2), 1340 cm^{-1} (ν_s SO_2). ^{1}H-NMR ($CDCl_3$) δ: 2.45 (3H, s, –CH_3), 4.89 (2H, s, –NH_2), 7.32 (2H, d, J = 8.0 Hz, C_3 and C_5 benzene H), 7.84 ppm (2H, d, J = 8.0 Hz, C_2 and C_6 benzene H). MS *m/z*: 171 (M^+), 155 ($M^+ - NH_2$), 91 ($M^+ - SO_2NH_2$). Products **2** and **3** were identical with authentic samples of 3-azabicyclo[3.3.0]oct-3-yl-amine monohydrochloride and *p*-toluenesulfonamide, respectively, in terms of mp, UV, IR and ^{1}H-NMR spectra and MS.

Binding of Gliclazide with Serum Proteins——Gliclazide binding with serum proteins was studied by equilibrium dialysis[9] in 0.067 M phosphate buffer (pH 7.4). A 2-ml portion of gliclazide solution (10 μg/ml) containing albumin (5 mg/ml), α-globulin (5 mg/ml), β-globulin (25 mg/ml) or γ-globulin (25 mg/ml) was taken into a dialysis bag (cellulose tubing, 20/32 inch inflated diameter, Visking Co., U.S.A.) which was placed in a glass vial (inner volume: 20 ml) containing 8 ml of gliclazide solution at the same concentration as that inside the bag. After continuous shaking of the vial at 120 strokes per min for 7 h at 37 °C in a water bath, the concentration of gliclazide outside the bag was measured by HPLC.[7]

Results and Discussion

Two degradation products of gliclazide were isolated from the reaction mixture and crystallized, yielding colorless plates (mp 180 °C) and colorless needles (mp 138 °C). They were identified as a hydrazine compound (**2**, 3-azabicyclo[3.3.0]oct-3-yl-amine monohydrochloride) and *p*-toluenesulfonamide (**3**) from the results of mp, UV, IR, ^{1}H-NMR, MS and HPLC studies.

As shown in Table I, the extent of gliclazide degradation was low (less than 12%) even at a high concentration (0.55 M) of phosphate ion. Sodium and potassium ions also did not affect gliclazide degradation. The degradation was less than 12% at a high level (989 meq/l) of sodium or potassium ion, indicating that phosphate buffer could be used to study anion-induced gliclazide degradation.

The effects of halide, sulfate, phosphate, carbonate and acetate ions on gliclazide degradation were examined in aqueous solution or 0.067 M phosphate buffer (pH 7.4) at 37 °C for 24 h (Fig. 1). The degradation of gliclazide reached 100% with chloride ion (0.2 M) and bromide ion (0.5 M). Fluoride, sulfate, acetate, carbonate and phosphate ions gave slight

Table I. Effects of Potassium and Sodium Ions on Gliclazide Degradation

Phosphate (M)	K^+ (meq/l)	Na^+ (meq/l)	Degradation rate (%)
0.07	121		10
0.10	175		8
0.55	989		12
0.07		121	10
0.10		175	10
0.19		338	12
0.46		827	9
0.55		989	10

Phosphate buffer solution containing various concentrations of potassium and sodium ions was prepared using monobasic potassium (or sodium) phosphate and dibasic potassium (or sodium) phosphate. The reaction was performed using 100 μg/ml gliclazide solution at 37 °C for 24 h. Each result is the mean value of 1—3 experiments.

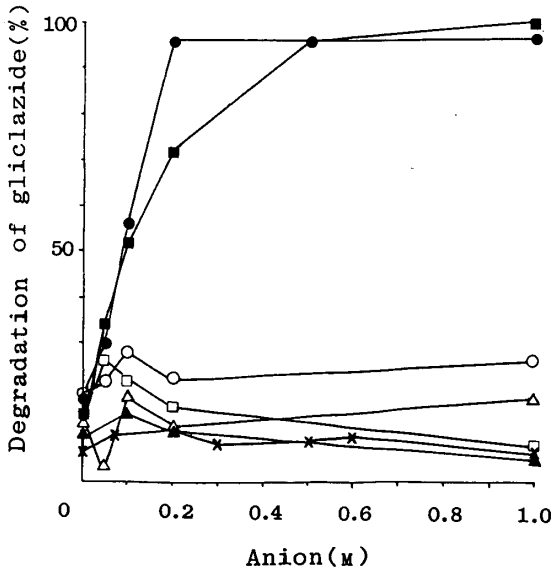
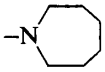
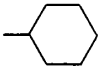
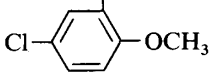
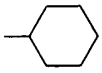


Fig. 1. Effects of Anions on Gliclazide Degradation

The effect of carbonate, acetate or sulfate ion on gliclazide degradation was examined in aqueous solution containing various concentrations of sodium carbonate, sodium acetate or sodium sulfate. The effects of halide ions were examined in 0.067 M phosphate buffer (pH 7.4) containing various concentrations of potassium fluoride, bromide or chloride. After incubation at 37 °C for 24 h, the concentration of intact gliclazide was determined. Each point is the mean of 2—6 experiments. (▲), F^-; (●), Cl^-; (■), Br^-; (○), CH_3COO^-; (△), CO_3^-; (□), SO_4^{2-}; (×), PO_4^{3-}

TABLE II. Relationship between Anion-Induced Degradation and Sulfonylurea Structure

Sulfonylurea	R_1	R_2	Degradation (%)
Gliclazide	$-CH_3$		84
Glycopyramide	$-Cl$		99
Tolazamide	$-CH_3$		92
Tolbutamide	$-CH_3$	$-CH_2(CH_2)_2CH_3$	0
Chlorpropamide	$-Cl$	$-CH_2CH_2CH_3$	0
Acetohexamide	$-COCH_3$		0
Glibenclamide	$-CH_2CH_2NHCO$ 		4

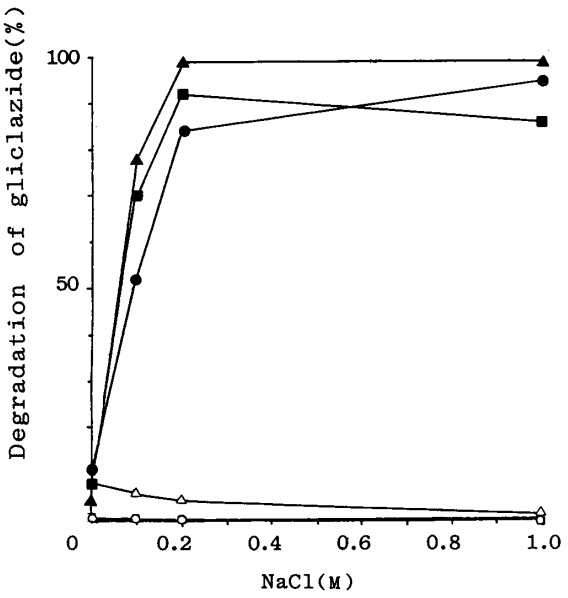


Fig. 2. Chloride-Induced Degradation of Sulfonylureas

Each point is the mean of 2—4 experiments. (●), gliclazide; (▲), glycopyramide; (■), tolazamide; (△), glibenclamide; (□), acetohexamide; (○), chlorpropamide.

gliclazide degradation (less than 25%), which was independent of the concentration of the anions.

Huck demonstrated that hydrazine and sulfonamide compounds were produced from glipizide, 1-cyclohexyl-3-[[*p*-[2-(5-methylpyrazinecarboxamido)ethyl]phenyl]sulfonyl]urea, by acid hydrolysis, suggesting that the reaction proceeded *via* protonation of amine nitrogen.[10] Although the degradation mechanism was not determined in the present study, this result seems to show that the degradation rate is related to the nucleophilic strength and leaving ability of the halide.[11] Other sulfonylureas such as tolbutamide, acetohexamide, chlorpropamide and glibenclamide were not degraded even in medium containing 1.0 M chloride ion. On the other hand, tolazamide and glycopyramide were degraded to extents of over 90%, like gliclazide (Fig. 2, Table II). Thus, the anion-induced degradation was specific for tolazamide, glycopyramide and gliclazide within the sulfonylurea group, and a character-

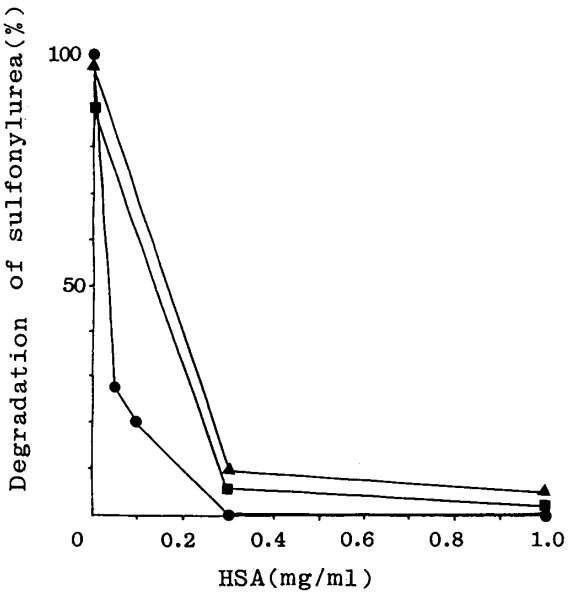


Fig. 3. Protective Effects of Human Serum Albumin on Chloride-Induced Degradation of Sulfonylurea

Sulfonylurea (100 μg/ml) was incubated in 0.067 M phosphate buffer (pH 7.4) containing 1.0 M sodium chloride at 37 °C for 24 h. Each point is the mean of 4 experiments. (●), gliclazide; (▲), glycopyramide; (■), tolazamide.

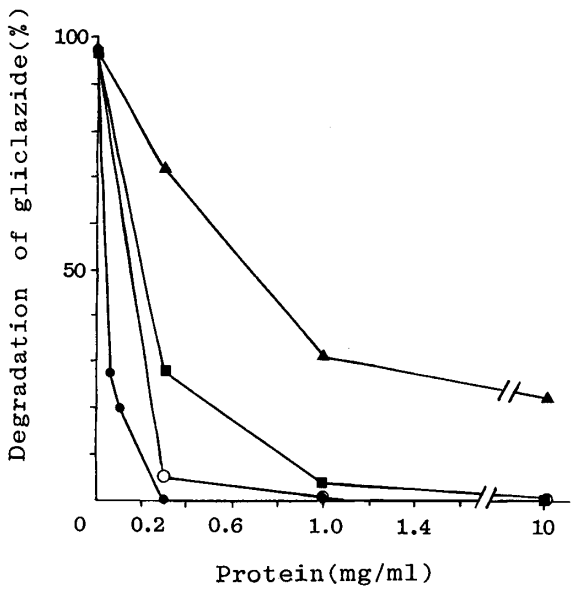


Fig. 4. Protective Effects of Serum Proteins on Chloride-Induced Gliclazide Degradation

Gliclazide (100 μg/ml) was incubated in 0.067 M phosphate buffer (pH 7.4) containing 1.0 M sodium chloride and various concentrations of serum proteins at 37 °C for 24 h. Each point is the mean of 2 experiments. (●), albumin; (○), α-globulin; (■), β-globulin; (▲), γ-globulin.

istic –CONH–aza-alicyclic structure seems to be required. A possible explanation for the faster degradation of hydrazine-containing sulfonylureas, such as gliclazide, is easier protonation of the hydrazine nitrogen. The present result seems to be analogous to our previous findings that the latter group of sulfonylureas, tolazamide, glycopyramide and gliclazide, has a characteristic inhibitory activity against glucose-, choline- and cholesterol-oxidase, whereas the former group of sulfonylureas, tolbutamide, acetohexamide, chlorpropamide and glibenclamide does not.[12]

Study of the protective effect of serum proteins on the anion-induced gliclazide degradation showed that the chloride ion (1.0 M)-induced gliclazide degradation decreased with increasing HSA concentration, and complete protection was observed at over 0.3 mg/ml of HSA (Fig. 3). Similarly, less than 10% glycopyramide or tolazamide was degraded in the presence of 1 mg/ml of HSA. Degradation induced by other halide ions such as bromide was also completely prevented at over 0.3 mg/ml of HSA.

As shown in Fig. 4, albumin among the four serum proteins offered the most protection against chloride (1.0 M)-induced gliclazide degradation, with complete protection provided by 0.3 mg/ml of HSA. The order of protective potency was albumin > α-globulin > β-globulin > γ-globulin.

It is well known that serum protein influences chemical reactions; the hydrolysis of melphalan (a nucleic acid-alkylating agent)[13] or chlorambucil (a nitrogen mustard anticancer agent)[14] is retarded by bovine serum albumin or human serum proteins. Moreover, chloride ion reduced the binding of warfarin to HSA at a physiological pH.[15] These findings suggest that the effect of serum proteins on drug hydrolysis is due to interaction or binding with the drug.

Two mechanism may be considered for the protective effects of serum proteins on the anion-induced gliclazide degradation. One is the binding of anions with serum protein resulting in a decrease of free anion,[16] and the other is interaction or binding of gliclazide

TABLE III. Gliclazide Binding with Serum Proteins

Serum protein (mg/ml)	Gliclazide/protein (μg/mg)	% binding
Albumin (5.0)	2.75	27.5
α-Globulin (5.0)	2.10	21.0
β-Globulin (25.0)	0.20	10.0
γ-Globulin (25.0)	0.15	7.5

Each result is the mean value of 2 experiments. Each reaction mixture contained 100 μg of gliclazide (10 μg/ml).

with serum proteins such as albumin or globulins. However, the former is not significant in the present study because the amount of chloride ion which is trapped by the serum proteins is too small to produce an inhibitory effect on the degradation of gliclazide.[13] On the other hand, as shown in Fig. 3, the degradation of gliclazide was completely prevented in the medium containing a low concentration of HSA (0.3 mg/ml), which is equivalent to 0.86% of normal serum albumin (35 mg/ml). This albumin concentration is too low to give complete binding of gliclazide (Table III). Therefore, neither of the above mechanisms, anion-trapping and drug-binding to serum proteins, seems to account for the observed effects. The true mechanism remains to be identified.

Based on the present results, it is considered that gliclazide may be degraded in the blood of patients with serious hypoproteinemia or hypoalbuminemia, but may not be degraded in usual diabetic patients who receive the drug orally.

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