J.Pharm.Dyn., **4**, 217—225 (1981)

DETERMINATION OF A NEW HYPOGLYCEMIC DRUG, GLICLAZIDE, IN HUMAN SERUM BY RADIOIMMUNOASSAY

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(Received September 20, 1980)

Radioimmunoassays have been developed which enable accurate and sensitive determination of gliclazide in human serum. Antisera A and B against gliclazide were obtained from guinea pigs immunized with conjugates A and B prepared by coupling gliclazide homologues, 1-(*p*-toluenesulfonyl)-3-(4′-carboxypiperidino)urea and 1-(4-methyl-3-carboxybenzenesulfonyl)-3-(3-azabicyclo[3,3,0]oct-3-yl)urea, to bovine serum albumin. ^{3}H-Gliclazide was used as a tracer. Dextran-coated charcoal was used to separate bound and free ^{3}H-gliclazide in the reaction mixture. The assays of gliclazide in serum were possible over a concentration range from 0.25 to 20 μg/ml with the antiserum A and from 0.1 to 10 μg/ml with the antiserum B, respectively, using 0.01 ml of human serum without the need for an extraction procedure. The antisera used for the assays were specific for gliclazide. Data obtained by the radioimmunoassay with the antiserum A are in good agreement with those by the radioimmunoassay with the antiserum B and gas–liquid chromatography. Serum levels of gliclazide in healthy volunteers receiving single oral dosing (40 mg/subject) have also been determined.

Keywords—radioimmunoassay; gliclazide; hypoglycemic drug; cross-reactivity of gliclazide antiserum; gliclazide level in human serum; gliclazide homologue-BSA conjugate

Gliclazide, N-(4-methylbenzenesulfonyl)-N′-(3-azabicyclo[3,3,0]oct-3-yl)urea (Fig. 1) is a new oral hypoglycemic drug having sulfonylurea structure.[1] For the rational clinical use of the drug, it seems desirable to monitor the blood levels of the unchanged drug.[2]

The gas chromatographic method developed by Bechtel *et al.*[2] and the high-performance liquid chromatographic method recently developed by Kimura *et al.*[3] permit the specific determination of gliclazide in plasma but are not highly sensitive and in addition the methods involve tedious extraction procedures. A more simple and sensitive method is, therefore, preferable for the routine determination of the unchanged drug in plasma or serum.

In this paper we describe simple radioimmunoassays with a high sensitivity and specificity for gliclazide in unextracted human serum, which are based on the use of specific antisera elicited with hapten-bovine serum albumin (BSA) conjugates A and B (Fig. 1). The serum levels of gliclazide in healthy volunteers are also described.

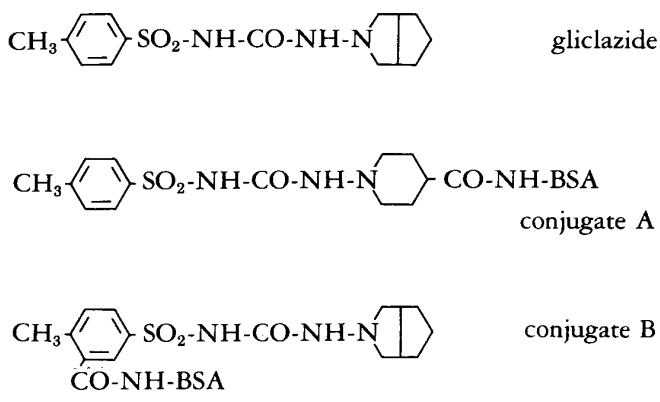

FIG. 1. *Structures of Gliclazide and Antigens*
BSA: Bovine serum albumin.

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EXPERIMENTAL

Chemicals and Reagents— ^{3}H-Gliclazide (labeled at the 3-position of the 4-methylbenzene moiety) with a specific activity of 113 mCi/mmol was synthesized by the catalytic reduction of the corresponding chlorinated derivative and the radiochemical purity was checked by thin-layer chromatography prior to use. Acetohexamide, glibenclamide, tolbutamide and chlorpropamide were extracted with solvent from their commercial preparations and were purified. Gliclazide was supplied by Les Laboratories Servier (Suresnes, France) and buformin was obtained from Dainippon Pharmaceutical Co. (Osaka). BSA (crystallized and lyophilized) was purchased from Sigma Chemical Co. (St. Louis, Mo.), Freund's complete adjuvant from Difco Laboratories (Detroit, Mich.), dextran T-70 from Pharmacia Fine Chemicals (Uppsala, Sweden), activated carbon (Norit A) from Nutritional Biochemical Corp. (Cleveland, Ohio), human control serum from Flow Laboratories (Rockville, Md.) and Riafluor® scintillation cocktail from New England Nuclear (Boston, Mass.). All other chemicals used were of analytical reagent grade.

Synthesis of Metabolites — N-(4-Carboxybenzenesulfonyl)-N'-(3-azabicyclo[3,3,0]oct-3-yl)urea (metabolite 1) was prepared according to the procedure of Beregi *et al.*,[4] mp 197° (dec.).

N-(4-Hydroxymethylbenzenesulfonyl)-N'-(3-azabicyclo[3,3,0]oct-3-yl)urea (metabolite 2) was prepared from 4-sulfamoylbenzyl alcohol[5] and 1,1-diphenyl-3-(3-azabicyclo[3,3,0]oct-3-yl)-urea[4] by the same method as described above, mp 164—166° (dec.). *Anal.* Calcd for $C_{15}H_{21}N_3O_4S$: C, 53.08; H, 6.24; N, 12.38; S, 9.45. Found: C, 52.83; H, 6.21; N, 12.36; S, 9.17.

Preparation of Immunogens

Immunogens were prepared as shown in Chart 1.

Synthesis of 1-(p-Toluenesulfonyl)-3-(4'-ethoxycarbonylpiperidino)urea [II] — II was prepared by applying the method of Wright *et al.*[6] A solution of I (7.3 g) in 10 ml of water was made alkaline with 5% $NaHCO_3$ and shaken with 40 ml of ether. The ether layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give 5.4 g of an oily substance [I]-base. [I]-base (5.4 g) and *p*-toluenesulfonylethylurethane[7] (7.3 g) were dissolved in 70 ml of absolute toluene. After refluxing for 2 hr, the reaction mixture was concentrated *in vacuo* to about 30 ml and cooled. The resultant precipitate was collected by filtration, washed with ether, and recrystallized from toluene–ether to give 7.7 g of II, mp 163—165°. *Anal.* Calcd for $C_{16}H_{23}N_3O_5S$: C, 52.02; H, 6.28; N, 11.37; S, 8.68. Found: C, 52.15; H, 6.39; N, 11.42; S, 8.90.

Synthesis of 1-(p-Toluenesulfonyl)-3-(4'-carboxypiperidino)urea [III] — To a solution of II (7.2 g) in 70 ml of dioxane was added 140 ml of 7.3% Na_2CO_3. After refluxing for 1.5 hr, the reaction mixture was concentrated *in vacuo* to about 100 ml. The resultant solution was brought to pH 3.0 with conc. HCl under cooling with ice-water. The precipitate was collected by filtration, washed with water, dried and recrystallized from dioxane-CH_3CN to give 4.8 g of III, mp 204—205° (dec.). *Anal.* Calcd for $C_{14}H_{19}N_3O_5S$: C, 49.26; H, 5.61; N, 12.31; S, 9.39. Found: C, 49.41; H, 5.61; N, 12.59; S, 9.58.

Synthesis of 1-(p-Toluenesulfonyl)-3-[4'-(2,5-dioxopyrrolidine-1-yl)oxycarbonylpiperidino]urea [IV] — To a suspension of III (1.0 g) in 10 ml of absolute CH_2Cl_2 was added 0.98 ml of triethylamine at room temperature with stirring. The reaction mixture was allowed to stand for 1 hr under cooling, to which 0.66 ml of isopropyl chloroformate was added. After 2 hr a solution of N-hydroxysuccinimide (674 mg) in 1 ml of dimethylformamide was added dropwise at 5—10° and allowed to stand for 1 hr. The resultant precipitate was collected by filtration, washed successively with cold CH_2Cl_2, cold water and cold acetone, dried and recrystallized from CH_3CN to give 500 mg of IV, mp 196—197° (dec.). *Anal.* Calcd for $C_{18}H_{22}N_4O_7S$: C, 49.31; H, 5.06; N, 12.78; S, 7.31. Found: C, 49.23; H, 5.00; N, 13.06; S, 7.44.

Preparation of Conjugate A — To a solution of BSA (800 mg) in 180 ml of 0.04 M phosphate buffer (pH 7.0) was added dropwise a solution of

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the ester IV (322 mg) in 45 ml of absolute dioxane at room temperature with stirring. After being allowed to stand for 4 hr, the reaction mixture was dialyzed against running water for about

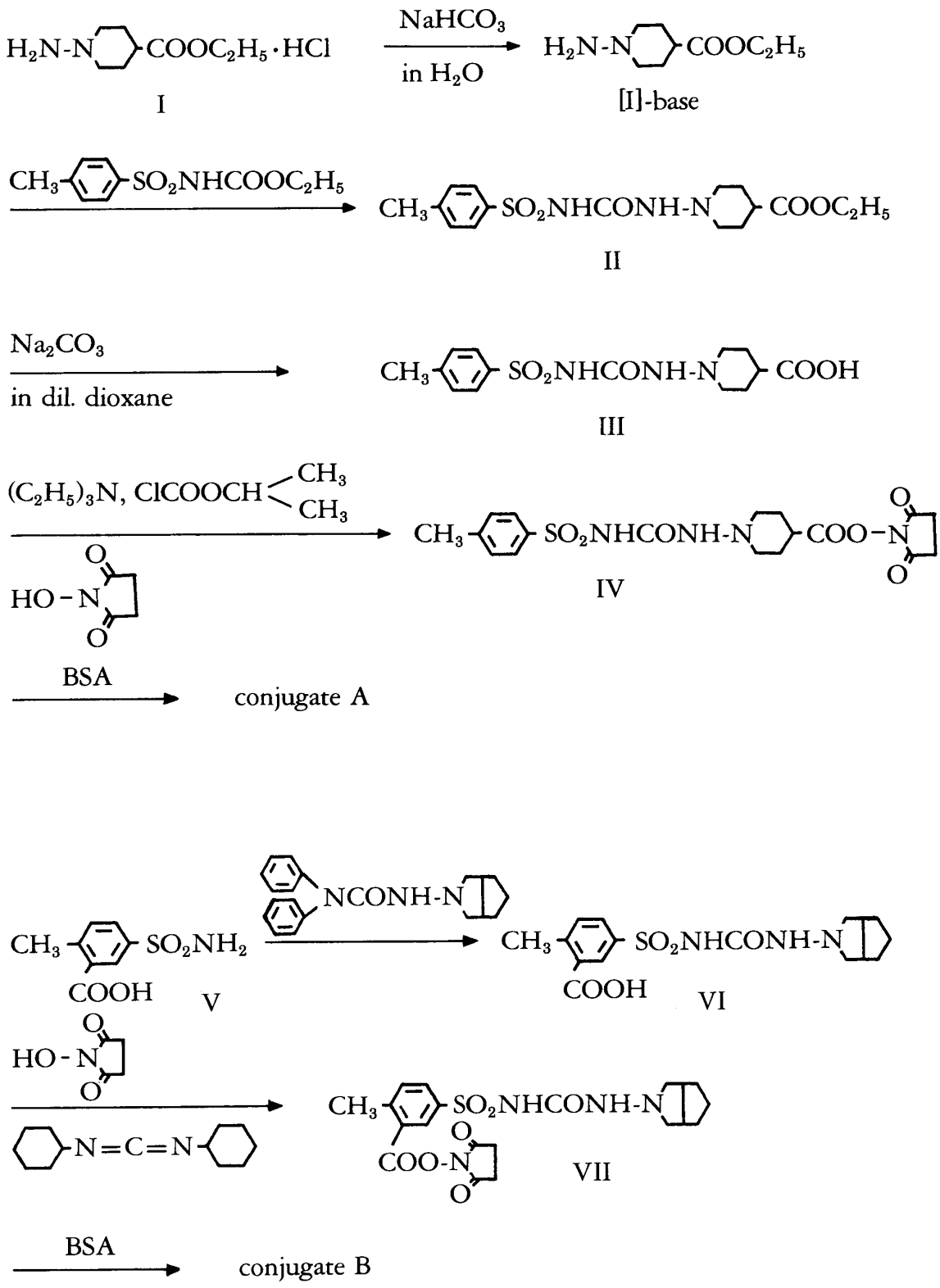

CHART 1

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16 hr and concentrated by ultrafiltration (PM-30, Amicon Corp., Lexington, Mass.) to about 80 ml. The resultant solution was brought to pH 4.5 with 0.5 N HCl and allowed to stand for 1 hr in refrigerator. The precipitate was collected by centrifugation and again suspended in 20 ml of water and brought to pH 8.5 with 0.25% NaOH to dissolve the precipitate. The solution was passed through a column of Sephadex G-25 and lyophilized to give fluffy powder.

Spectrophotometric analysis of the conjugate against BSA and [III]-$NHCH_3$ {*1-(p*-toluenesulfonyl)-3-[4′-(N-methylcarbamoyl)piperidino]urea} indicated that about 20 mol of [III] had been covalently coupled to 1 mol of BSA.

Synthesis of 1-(4-Methyl-3-caboxybenzenesulfonyl)-3-(3-azabicyclo[3,3,0]oct-3-yl)urea [VI] — To 2-methyl-5-sulfamoylbenzoic acid[8] (3.8 g) in 20 ml of dimethylformamide was added dropwise 8 ml of 25% KOH at room temperature. After stirring for 15 min at 50—70°, a solution of 1,1-diphenyl-3-(3-azabicyclo[3,3,0]oct-3-yl)urea (6.0 g) in 40 ml of dimethylformamide was added dropwise. The reaction mixture was heated for 1 hr in boiling-water bath and evaporated *in vacuo*. The residue dissolved in 70 ml of water was shaken with ether. The aqueous layer was adjusted to pH 3.5 with 1N HCl in ice-water bath. The precipitate was collected by filtration, washed with water and recrystallized from dioxane-ethyl acetate to give 2.4 g of VI, mp 190—191° (dec.).*Anal.* Calcd for $C_{16}H_{21}N_3O_5S$: C, 52.30; H, 5.76; N, 11.44; S, 8.73. Found: C, 51.81; H, 5.88; N, 11.17; S, 8.30.

Synthesis of 1-[3-(2,5-Dioxopyrrolidine-1-yl)oxycarbonyl-4-methylbenzenesulfonyl]-3-(3-azabicyclo-[3,3,0]oct-3-yl)urea [VII] — An ester [VII] was prepared from VI and N,N′-dicyclohexylcarbodiimide by the conventional method. mp 196—202°(dec.). *Anal.* Calcd for $C_{20}H_{24}N_4O_7S$: C, 51.72; H, 5.21; N, 12.06; S, 6.90. Found: C, 52.86; H, 5.69; N, 11.76; S, 6.39.

Preparation of Conjugate B — The conjugate B was prepared by the same method as described in Preparation of Conjugate A. Spectrophotometric analysis of the conjugate against BSA and [VI]-$NHCH_3$ {1-3-(N-methylcarbamoyl)-4-methylbenzenesulfonyl]-3-(3-azabicyclo[3,3,0]oct-3-yl)-urea} indicated that about 25 mol of VI had been covalently coupled to 1 mol of BSA.

Immunization

Three male Hartley guinea pigs weighing 350—450 g were immunized with each of the two conjugates. Each guinea pig was given 1 mg of the conjugate, which was dissolved in 0.5 ml of sterile isotonic saline and emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected into the guinea pig intradermally at several sites over the back and in the foot pads. Booster injections were given every two weeks for 6 weeks and then every four weeks for additional three months. Blood was collected by cardiac puncture 7 days after each or the final booster injections, and the serum was separated and stored at –20°.

Assay Procedure

The following solutions were prepared prior to assay: (1) 0.075 M phosphate buffer at pH 7.4 (assay buffer), (2) a solution of ^{3}H-gliclazide in the assay buffer to give 1.1 × 10^4 dpm/15 ng/0.05 ml, (3) various antiserum dilutions with the assay buffer, (4) standard solutions containing 0, 1, 2.5, 5, 10, 20, 40, 60, 100 and 300 ng of unlabeled gliclazide in 0.4 ml of the assay buffer, and (5) dextran-coated charcoal (DCC) suspension containing 2 g of activated charcoal and 200 mg of dextran T-70 in 100 ml of the assay buffer.

Antiserum titer was determined by adding 0.1 ml of various antiserum dilutions to an incubation mixture consisting of 0.05 ml of ^{3}H-gliclazide solution, 0.01 ml of control serum and 0.5 ml of an assay buffer containing 10% of control serum in 1.5 ml of Eppendorf plastic tubes. Normal guinea pig serum was used as a blank. The contents were vortexed and incubated at room temperature for 2 hr with continuous rotation by Immunorotor(25 rev/min, Daiichi Radioisotope Lab. Ltd., Tokyo). To separate antibody-bound and free drug, 0.1 ml of DCC suspension was added to each tube. The tubes were vortexed, stood at 4° for 10 min and centrifuged at 3500

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rpm for 10 min. One-half milliliter of the supernatant was pipetted into a counting vial containing 10 ml of Riafluor scintillation cocktail and the radioactivity was counted in a scintillation spectrometer (Packard Tri-Carb Model 2450). The percentage of antibody-bound ^{3}H-gliclazide to the total count initially added was calculated and plotted against the final dilution of antiserum.

Each standard curve was obtained by adding various amounts of gliclazide ranging 1—300 ng/tube to the assay system and by plotting the relative percentage (B/B_0)[9] of bound labeled drug against the amount of unlabeled gliclazide added on a logit-log paper.

In order to assay unknown serum samples, 0.01 ml of serum samples was added to the assay system instead of 0.01 ml of control serum. All samples were run in duplicate.

Antibody specificity was evaluated by measuring the inhibition of the antibody-gliclazide binding caused by increasing amounts of the selected compounds including the metabolites[10] of gliclazide.

Gas-Liquid Chromatography

A JEOL, model JGC-20K, gas chromatograph equipped with a flame ionization detector was used. A silanized glass column (100 cm × 2 mm I.D.) was packed with 3% XE-60 on Chromosorb W AWDMCS (80—100 mesh). Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. The column temperature was 220° and the injector and detector temperatures were 280°.

Gliclazide in serum is extracted with chloroform and, after a clean-up procedure, derivatized with diazomethane followed by heptafluorobutyric anhydride to form N-methyl-N′-heptafluorobutyryl gliclazide, which is assayed on a gas chromatograph. Further details of the assay will be reported elsewhere.

Human Study

The study was carried out under the supervision of physicians. Two healthy volunteers each received 40 mg of gliclazide in tablet form, and blood was taken at 1, 2, 3, 4, 6, 8, 12 and 24 hr after dosing. The sera were separated and kept

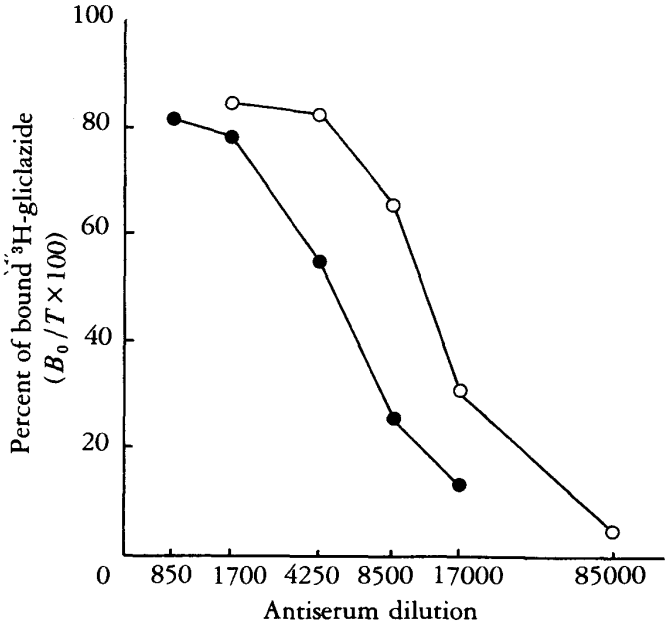

FIG. 2. *Dilution Curves of Gliclazide Antisera*
The percentage of ^{3}H-gliclazide (15 ng) bound to the antiserum A (●) or B (○) is plotted against the final dilution of the antisera presented on a semi-logarithmic scale.

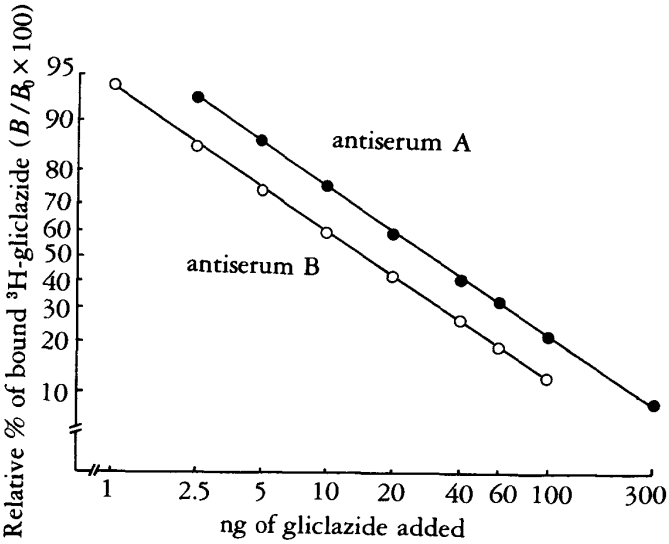

FIG. 3. *Standard Curves of Gliclazide*
The relative percentage of ^{3}H-gliclazide bound to the antiserum A or B in the presence of various amounts of unlabeled gliclazide is plotted on a logit-log scale.

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frozen until analyzed.

RESULTS

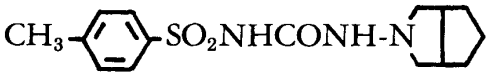
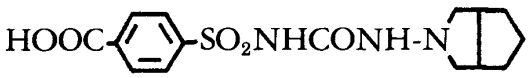
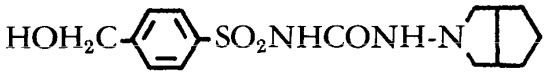
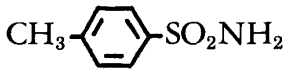
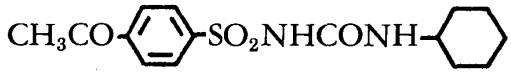
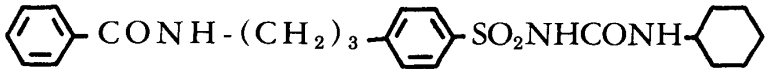
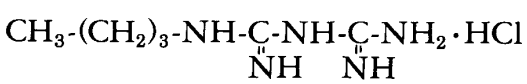
Antibody Titer

All six of the guinea pigs immunized with the conjugate A or B produced useful antisera (A and B). Typical titer curves of the antisera are shown in Fig. 2. The sharp slope of the curves indicates a high affinity of the antibodies to gliclazide. The dilutions of the antisera A and B chosen for the assay were 1:5000 and 1:12500 in final dilution, respectively, which gave about 50% binding.

Sensitivity of the Assay

Typical standard curves obtained with antisera and 15 ng of the labeled tracer, for non-labeled gliclazide added to control human serum, are

TABLE I. *Specificity of Gliclazide Antisera*

Name	Structure	Cross-reactivity $X/Y \times 100\%$ Antiserum A	Antiserum B
Gliclazide		100	100
Gliclazide metabolite 1		<0.1	3.7
Gliclazide metabolite 2		2.7	90
p-Toluene-sulfonamide		<0.1	<0.1
Acetohexamide		1.3	13.8
Glibenclamide		<0.1	0.2
Tolbutamide		6.6	0.2
Chlorpropamide		13.5	<0.1
Buformin		<0.1	<0.1

The specificity of the antisera A and B was evaluated by incubating the antiserum A (× 5000, 0.1 ml) or B (× 12500, 0.1 ml) and ^{3}H-gliclazide (15 ng) with the test compound, in the amounts ranging 1 to 10000 ng per assay tube. The values are expressed in percentage cross-reactivity which is defined as X/ Y× 100, where X is the amount of unlabeled gliclazide and Y is the amount of the test compound required to produce 50% inhibition of binding of ^{3}H-gliclazide to the antisera.

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shown in Fig. 3. Gliclazide in 0.01 ml of serum can be reliably assayed in a range of 2.5—200 ng with the antiserum A and 1—100 ng with the antiserum B.

Intra and Inter-Assay Variations

Intra and inter-assay variations of the radioimmunoassays with the antisera A and B were estimated from the repeated assay of human pooled sera. The coefficients of variation were less than 10% over the concentrations tested.

Specificity of the Antiserum

The cross-reactivity of the antisera with the selected compounds is listed in Table I. Data are expressed in terms of percentage cross-reactivity. None of the compounds showed an appreciable cross-reaction with the antiserum A except for chlorpropamide (13.5%) and tolbutamide (6.6%). While hydroxymethyl gliclazide (metabolite 2) and acetohexamide showed a cross-reaction with the antiserum B of 90% and 13.8%, respectively. In plasma over 95% of the drug related compounds is reported to be unchanged in animals and man receiving gliclazide.[10] Therefore, the two assays should be specific for gliclazide in human serum except for a concomitant administration of chlorpropamide or acetohexamide.

Comparison of Two Radioimmunoassays with Antiserum A and Antiserum B

Eight human serum samples of gliclazide were determined by the radioimmunoassays with the antiserum A and the antiserum B. The results are shown in Fig. 4. There is a good correlation between values obtained by the two assays (correlation coefficient, 0.998).

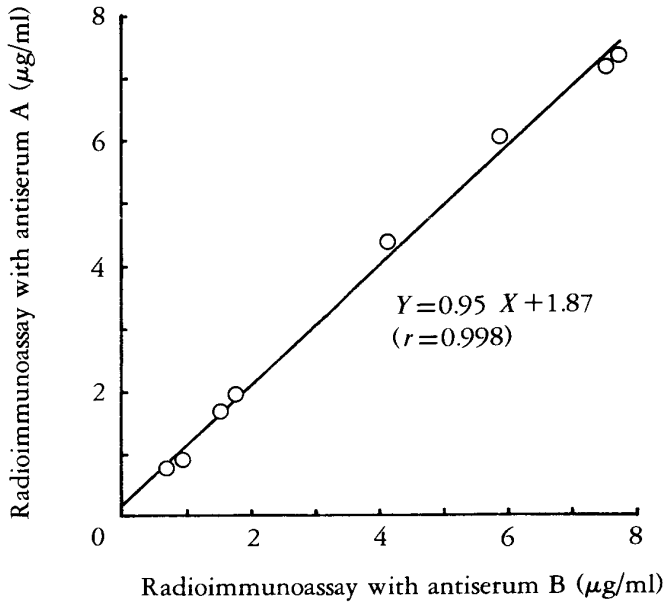

FIG. 4. *Comparison of Radioimmunoassays with Antiserum A and Antiserum B of Gliclazide in Human Serum*

Serum samples were obtained from volunteers after oral administration of gliclazide. Line was drawn from the values calculated by the method of least squares.
X: radioimmunoassay with antiserum B (μg/ml).
Y: radioimmunoassay with antiserum A (μg/ml).
r: correlation coefficient.

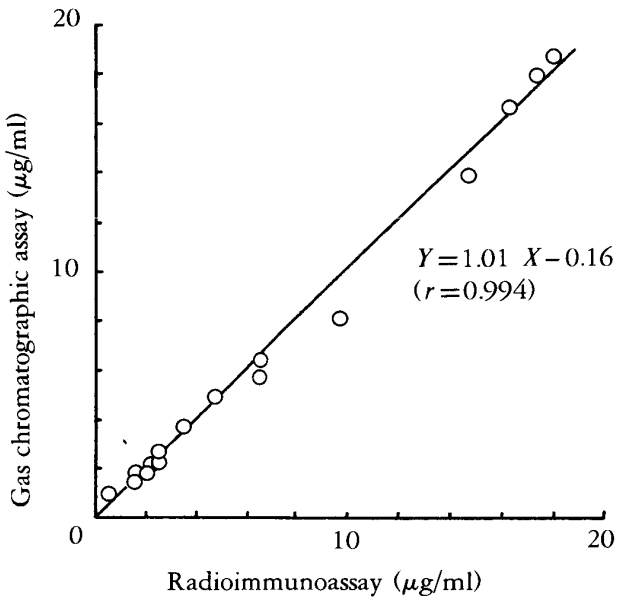

FIG. 5. *Comparison of Radioimmunoassay and Gas Chromatographic Assay of Gliclazide in Human Serum*

Serum samples were obtained from volunteers after oral administration of gliclazide. Line was drawn from the values calculated by the method of least squares.
X: radioimmunoassay with antiserum A (μg/ml).
Y: gas chromatographic assay (μg/ml).
r: correlation coefficient.

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Comparison of Radioimmunoassay and Gas-Liquid Chromatography

Sixteen human serum samples of gliclazide were determined by both radioimmunoassay with the antiserum A and gas-liquid chromatography. The results are shown in Fig. 5. A good correlation was found between values obtained by the two methods (correlation coefficient, 0.994).

Serum Levels of Gliclazide in Humans

Fig. 6 presents the serum levels of gliclazide in two male healthy volunteers each receiving orally 40 mg of gliclazide. The unchanged drug levels reached a maximum of about 3 μg/ml at 2—4 hr after dosing, and declined to half the peak level in 6.7—7.1 hr. These results are consistent with previous findings.[10]

DISCUSSION

Radioimmunoassays were developed for gliclazide in unextracted human serum with a high sensitivity and specificity. Accurate determinations of gliclazide are possible with 0.01 ml of serum samples over a concentration range of 0.25—20 μg/ml with the antiserum A and a concentration range of 0.1—10 μg/ml with the antiserum B.

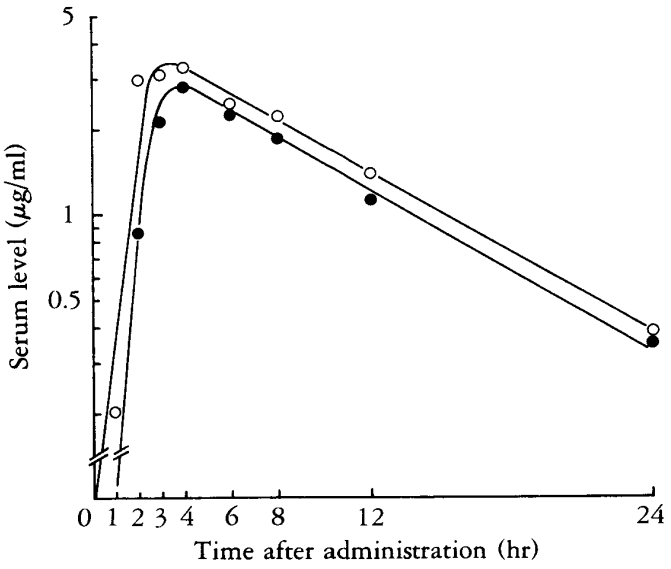

FIG. 6. *Serum Levels of Gliclazide in Two Volunteers after Single Oral Administration at a Dose of 40 mg/subject*

The antisera A and B were prepared by using the conjugates A and B (Fig. 1). The antiserum A elicited with the conjugate A, the hapten being coupled to BSA through 4-position of the piperidine moiety, did not show any appreciable cross-reaction with the selected compounds except for chlorpropamide (13.5%) and tolbutamide (6.6%) (Table I). While the antiserum B elicited with the conjugate B, the hapten being coupled to BSA at 3-position of the benzene ring, showed a significant cross-reaction with hydroxymethyl gliclazide (metabolite 2) and to some extents with acetohexamide (13.8%), but did not show any appreciable reaction with chlorpropamide and tolbutamide. The specificity of the antibodies, therefore, seems to be directed to the portion of the hapten molecule furthest from the site of conjugation to the carrier protein.

Serum levels of the unchanged drug in man receiving gliclazide were determined by each of the two radioimmunoassays. The values obtained by the assay with the antiserum A were almost equal to those obtained by the assay with the antiserum B (Fig. 4). These results suggested that the hydroxymethyl metabolite of gliclazide was scarcely present in the human serum, because the hydroxymethyl metabolite showed a cross-reaction with the antiserum A by 2.7% while the antiserum B by 90%, respectively.

The antisera A and B showed a cross-reaction with chlorpropamide, tolbutamide or acetohexamide to some extents (Table I). Sulfonylurea drugs are generally not concomitantly administered with each other and, therefore, the radioimmunoassays described here are substantially specific for gliclazide in human serum.

In addition, the radioimmunoassays offer distinct advantages, as compared to the gas chromatographic and high-performance liquid chromatographic methods; accurate and sensitive determination with a small volume of serum as little as 0.01 ml, in contrast to 4 ml of plasma needed for the gas chromatography[2] and 2.5 ml of plasma needed for the high-performance liquid

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chromatography,[3] and simple procedure with intact serum enabling to assay a large number of samples, in contrast to tedious extraction procedures in the high-performance liquid chromatography and tedious extraction and derivatization procedures in the gas chromatography.

The present radioimmunoassay procedure is simple and sensitive enough to allow for the determination of gliclazide with a small portion of serum remaining after insulin and glucose determinations, thereby, offering a potential means leading to a better control in the treatment of diabetics.

Acknowledgement The authors express their gratitude to Dr. H. Nishimura, Director of the Research Laboratories, for his continued encouragement.

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