Radioimmunoassay of Glibenclamide

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SUMMARY

A specific, sensitive, and simple radioimmunoassay was developed for the oral hypoglycemic drug glibenclamide, N-4-[2-(5-chloro-2-methoxy-benzamide)-ethyl]-benzenesulfonyl-N′-cyclohexylurea. Antiserum against glibenclamide was obtained from rabbits immunized with an antigen prepared by conjugating the diazonium salt of N-(*p*-amino-benzamidoethyl)-benzenesulfonyl-N′-cyclohexylurea to bovine serum albumin through the diazocoupling. [^{3}H]glibenclamide was used as a tracer. Dextran-coated charcoal was used to separate bound and free [^{3}H]glibenclamide in the reaction mixture. The radioimmunoassay is able to determine as little as 25 pg of glibenclamide directly in plasma without the need for extraction. The antiserum used for the assay was highly specific for glibenclamide, and did not cross-react with two known major metabolites of glibenclamide. Comparable values of glibenclamide in dog plasma were obtained by radioimmunoassay and liquid chromatography. Plasma concentrations of glibenclamide in diabetic patients on glibenclamide treatment can be determined by radioimmunoassay, and the method has been applied to the routine assay of clinical samples. This radioimmunoassay seems to be useful for monitoring plasma glibenclamide concentrations. DIABETES *28*:221–226, March 1979.

Glibenclamide, N-4-[2-(5-chloro-2-methoxy-benzamide)-ethyl]-benzenesulfonyl-N′-cyclohexylurea, is one of the oral hypoglycemic drugs containing the sulfonylurea structure. Because of its high potency and low plasma concentration, no useful method has been available for monitoring its plasma concentration in clinical samples.

Most of the studies on the pharmacokinetics and metabolism of glibenclamide have been performed with the labeled drug,[1] but this method is not applicable for the routine clinical assay of the drug. Methods currently used for the determination of glibenclamide are the spectrofluorometric method[2] and the liquid chromatography developed by Fujimoto et al. (personal communication), a modification of the procedure reported by Sved et al.[3] These methods require time-consuming extraction and purification before the final determination, and sometimes lack the desired sensitivity. As a consequence, there have been reports on development of radioimmunoassay, but the antisera used so far lacked the desired specificity because they cross-reacted with the two major metabolites of glibenclamide.[4,5,6]

This paper describes the production of a specific antiserum to glibenclamide in rabbits immunized with an antigen prepared by a novel method. The antiserum has been used for a specific, sensitive, and simple radioimmunoassay for glibenclamide in plasma.

MATERIALS AND METHODS

REAGENTS

Glibenclamide, tritiated [cyclohexyl-3,4-^{3}H]glibenclamide with a specific activity of 17.2 Ci/mmol, 4-*trans*-hydroxy derivative (M1) and 3-*cis*-hydroxy derivative (M2) of glibenclamide, and *p*-aminoethyl-benzenesulfonyl-N-cyclohexylurea were all supplied by Hoechst Aktiengesellschaft, Frankfurt, West Germany, and Hoechst Japan, Tokyo. Glipizide was supplied by Fujisawa Pharmaceutical, Osaka. We obtained *p*-nitro-benzoyl-chloride and palladium carbon (Pd-C) from Wako Pure Chemicals, Tokyo. Bovine serum albumin (BSA) was purchased from Sigma Chemical, St. Louis, Missouri. Lysozyme was obtained from P-L Biochemicals, Milwaukee, Wisconsin. Complete Freund's adjuvant was purchased from Difco Laboratories, Detroit, Michigan. Omnifluor was obtained from New England Nuclear, Boston, Massachusetts. Norit A was obtained from American Norit, Jacksonville, Florida. Dextran T70 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals and materials were purchased from standard

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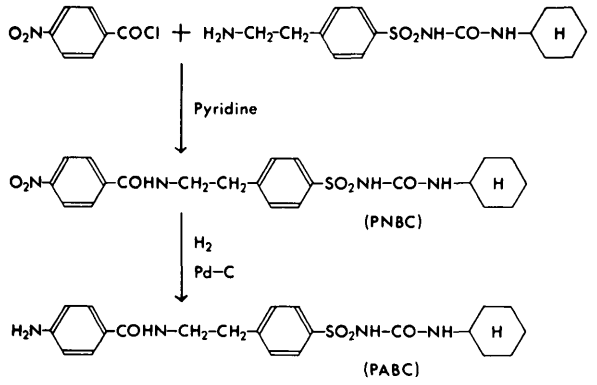
A. PREPARATION OF GLIBENCLAMIDE DERIVATIVES



B. PREPARATION OF GLIBENCLAMIDE IMMUNOGEN

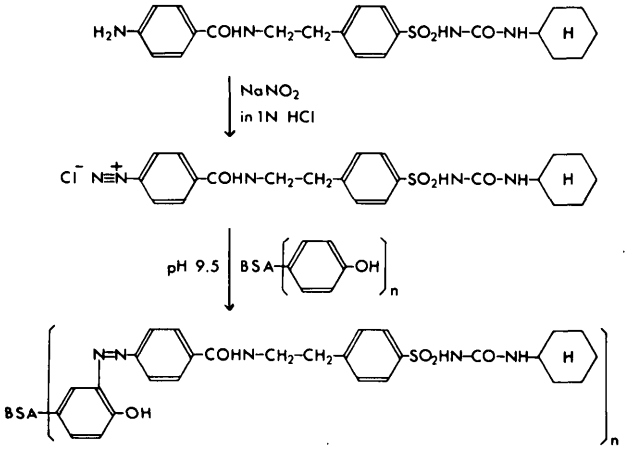


FIGURE 1. Procedures for the preparation of glibenclamide derivatives and immunogen. BSA, bovine serum albumin.

commercial sources. Phosphate-buffered saline (PBS) at pH 7.4 used for radioimmunoassay and immunogen dissolution consists of 8 g NaCl, 0.2 g KCl, 2.90 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4, 0.1 g merthiolate, and distilled water up to 1 L.

PREPARATION OF IMMUNOGEN

Synthesis of glibenclamide derivatives (Figure 1A). N-(*p*-nitrobenzamidoethyl)-benzenesulfonyl-N′-cyclohexylurea (PNBC). To a solution of *p*-aminoethyl-benzenesulfonyl-N-cyclohexylurea (108 mg, 0.33 mmol) in pyridine was added *p*-nitrobenzoyl chloride (62 mg, 0.33 mmol), with constant stirring at room temperature. After being stirred for 2 h, the reaction mixture was evaporated. The residue was washed with ether and recrystallized from ethanol. PNBC had a melting point of 215–217°C (elemental analysis of $C_{22}H_{26}N_4O_6S$; theoretical: C, 55.69; H, 5.53; N, 11.81%; found: C, 55.25; H, 5.53; N, 11.67%).

N-(*p*-aminobenzamidoethyl)-benzenesulfonyl-N′-cyclohexylurea (PABC). A solution of PNBC (39 mg, 0.08 mmol) in 10 ml of ethanol was hydrogenated in the presence of 5% Pd-C catalyst (13 mg). The mixture was stirred for 1 h at room temperature. The catalyst was removed by filtration and the filtrate was concentrated. The product was crystallized from ethanol. PABC had m.p. 197–201°C (elemental analysis of $C_{22}H_{28}N_4O_4S$; theoretical: C, 59.44; H, 6.35; N, 12.60%; found: C, 58.63; H, 6.16; N, 12.43%).

Conjugation of glibenclamide derivative to bovine serum albumin (Figure 1B). The procedures described below were performed at 0–4°C. To a solution of PABC (15 mg, 0.034 mmol) in 3 ml of 1 N HCl was added dropwise a solution of sodium nitrite (0.04 mmol) in water, with constant stirring. To remove the excess nitrous acid, a solution of sodium sulfamate (0.01 mmol) was added. The diazonium salt solution of PABC was added dropwise to 75 mg of bovine serum albumin (BSA) in 10 ml of water, and the pH of the solution was maintained around 9.5 with 0.5 N NaOH. The solution was stirred for 20 min after the addition of the final drop. After dialysis for 2 days against water, the immunogen was lyophilized. The conjugation of glibenclamide derivative to BSA was confirmed by the yellowish coloration of the immunogen. The number of haptenic molecules per BSA molecule was estimated as 10.3 on the basis of its absorbance at 240 nm in 0.01 N NaOH compared with the standard curves of BSA and PABC.

Immunization. Male albino rabbits were immunized with the immunogen once a week for 2 wk and then once every 2–4 wk. One milligram of the immunogen was dissolved in 0.5 ml of PBS and emulsified with an equal volume of complete Freund's adjuvant. One milliliter of the emulsion was injected into the four foot pads, intramuscularly into both thighs and subcutaneously into several sites on the back. Blood was collected from the central ear artery 1 wk after the fourth injection. Blood was allowed to clot for 2 h at room temperature and centrifuged at 3000 rpm for 15 min at 4°C to separate serum. Subsequent booster injections were given once every 4–6 wk.

Paper electrophoresis. One hundred microliters of PBS, normal rabbit, or human serum or rabbit antiserum was mixed with 50 μl of [^{3}H]glibenclamide (about 8 ng, 0.28 μCi) and incubated overnight at 4°C. In some tubes, 10 μl of unlabeled glibenclamide (500 μg/ml) was added to test the presence of competitive inhibition. Paper electrophoresis of the reaction mixture was performed on Toyo filter paper no. 51 with veronal buffer (pH 8.6, ionic strength 0.075) for 6 h at 7 mA. The dried paper strip was scanned for radioactivity by a paper chromatograph scanner (Aloka, JPC-213), and then stained with amido black to compare the distribution of radioactivity with that of serum proteins.

Radioimmunoassay. The radioimmunoassay of glibenclamide was performed in 10 × 75-mm glass tubes at 4°C. The antiserum was diluted with 0.1% lysozyme solution in PBS.

To determine the antiserum titer, 0.1 ml of various dilutions of antiserum was added to the incubation mixture consisting of 0.1 ml of [^{3}H]glibenclamide standard solution in PBS (approximately 130 pg, 10,000 dpm) and PBS to bring the volume up to 0.5 ml. The reaction almost reached equilibrium within 1 h, but for convenience, especially when a large number of samples is handled, the reaction was allowed to proceed overnight. The antibody-bound [^{3}H]glibenclamide was separated from free [^{3}H]glibenclamide by adding 0.5 ml of dextran-coated charcoal suspension (2.5% Norit A, 0.25% Dextran T70) and centrifuging at 3000 rpm for 10 min. The supernatant was decanted into a counting vial containing 9 ml of scintillation cocktail (toluene:Triton X-100

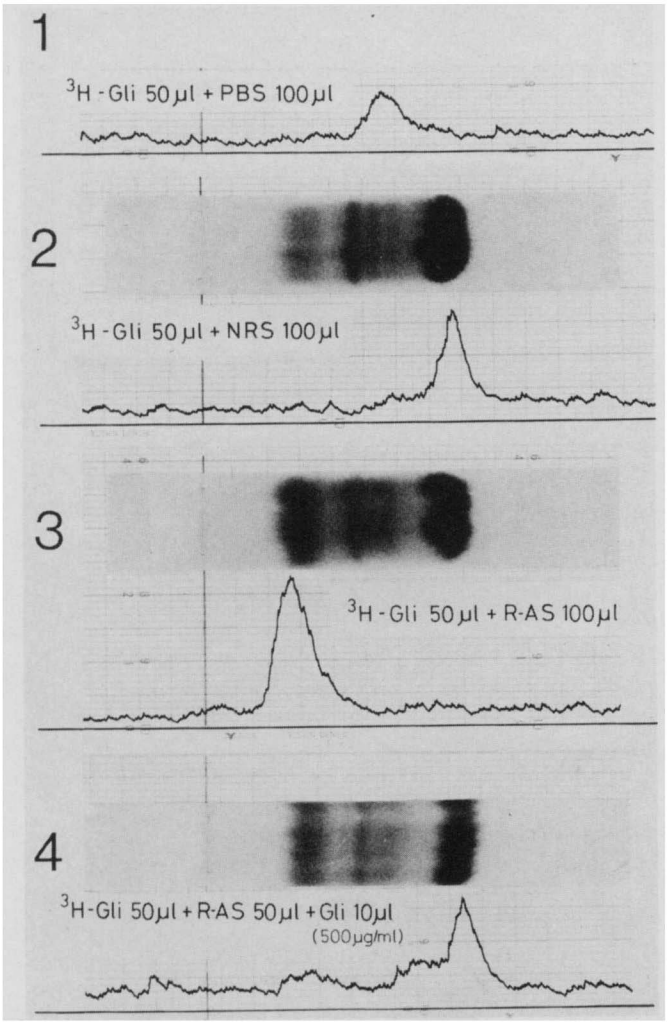
= 2:1, Omnifluor 4 g/L). The radioactivity was counted in a Packard Tri-Carb, Model 3390 liquid scintillation system. The percentage of antibody-bound [^{3}H]glibenclamide to the total count initially added was calculated and plotted against the final dilution of antiserum.

The specificity of the antiserum to glibenclamide, its metabolites, and a structurally related compound, glipizide, was evaluated by determining the amounts of each compound needed to inhibit [^{3}H]glibenclamide-antibody complex by 50%. The cross-reactivity of each compound was expressed as percentage (X/Y × 100) of the 50% inhibitory amount of glibenclamide (X) to the 50% inhibitory amount of the test compound (Y).

A standard curve was obtained by adding to the assay system various amounts of glibenclamide ranging from 25–5000 pg in 10 μl. Glibenclamide was dissolved in ethanol to make a stock solution of 500 μg/ml. Standard solutions were freshly prepared for each assay by diluting a stock solution with 0.1% lysozyme solution in PBS.

To assay unknown plasma samples, the same volume (usually 10 μl) of control plasma was added to the standard curve tubes as the unknown. Standards and samples were run in duplicate and results were calculated separately for each tube. The values for the unknown samples were read from the standard curve.

FIGURE 2. Demonstration of antibody to glibenclamide by paper electrophoresis. (1) In the absence of serum, [^{3}H]glibenclamide ([^{3}H]Gli) moves near α-β globulin region. (2) In the presence of normal rabbit serum (NRS), it moves together with albumin. (3) [^{3}H]glibenclamide is bound to γ-globulin region when incubated with rabbit antiserum (R-AS). (4) The binding is displaced by the addition of excess unlabeled glibenclamide (Gli).



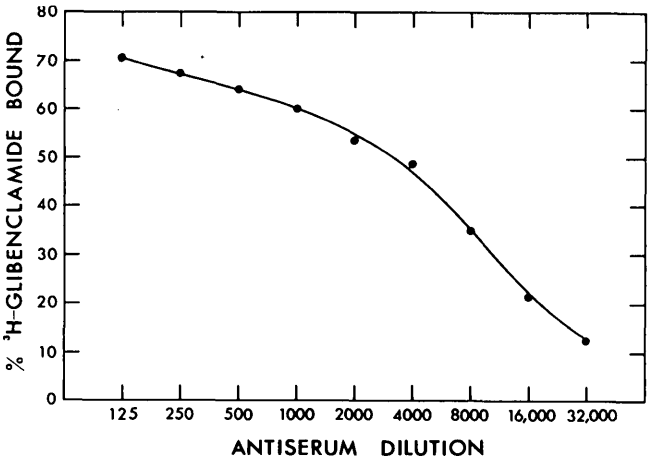


FIGURE 3. Glibenclamide antiserum dilution curve. The percentage of [^{3}H]glibenclamide (10,000 dpm, approximately 130 pg) bound to antibody is plotted against the final dilution of the antiserum represented on a semilogarithmic scale.

Dog and human plasma. Twenty-one plasma samples obtained from seven dogs were supplied from Hoechst Japan, Tokyo. Dogs with body weight of about 9 kg were bled at various intervals after oral administration of 12.5 mg glibenclamide. Because of the sensitivity of the radioimmunoassay, dog plasma was diluted 10 times with PBS and a 10-μl portion of the diluted sample (corresponding to 1 μl of the original plasma sample) was used for the assay.

Plasma samples were obtained from diabetic patients being treated with glibenclamide for at least 1 wk before blood sampling. A 10-μl portion of plasma was used for the radioimmunoassay.

RESULTS

Demonstration of antibody to glibenclamide by paper electrophoresis (Figure 2). The presence of antibody in the serum of rabbits after the fourth immunization was confirmed by a paper electrophoresis. With normal human or rabbit serum, [^{3}H]glibenclamide moved together with the albumin fraction. When [^{3}H]glibenclamide was incubated with the antiserum, the peak of radioactivity was found in the region corresponding to the γ-globulin fraction, and the addition of unlabeled glibenclamide displaced most of this radioactivity. The mobility of [^{3}H]glibenclamide without serum proteins corresponded to α-β globulin region.

Antibody titer. Figure 3 shows the percentage of [^{3}H]glibenclamide bound to various dilutions of antiserum. The dilution of the antiserum chosen for the radioimmunoassay was 1:8000 in final dilution, which gave about 35% binding.

Sensitivity of radioimmunoassay. Figure 4 indicates that as little as 25 pg of glibenclamide can be determined by this procedure. The standard curve is linear up to 5000 pg when plotted on a log-logit scale.

Specificity of radioimmunoassay. Two known major metabolites of glibenclamide found in man are the hydroxylated derivatives at C4 (M1) or at C3 (M2) of the cyclohexyl ring.[1] The cross-reactivity of these metabolites with the antiserum

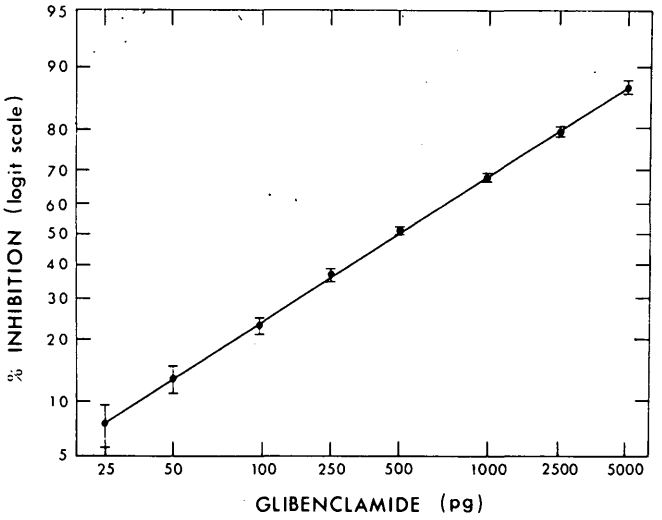


FIGURE 4. Glibenclamide radioimmunoassay standard curve. The inhibition of the binding of [^{3}H]glibenclamide to the antibody in the presence of various amounts of unlabeled glibenclamide is plotted on a log-logit paper. Each point represents mean and SD for four assays.

was found to be no more than 0.35% (Figure 5). Thus, the radioimmunoassay has a specificity for glibenclamide and the major metabolites do not interfere with the assay. Glipizide, a closely related oral hypoglycemic drug, showed a significant cross-reactivity (41.7%).

Comparison of radioimmunoassay and liquid chromatography. The same dog plasma samples that had been analyzed by liquid chromatography at another laboratory (Personal communication from Dr. K. Fujimoto. The samples were assayed by the method of Sved et al.[3] with some modifications.) were determined for glibenclamide by the radioimmunoassay. Because of the lack of sensitivity of the liquid chromatographic procedure, the comparison was performed over a concentration range of 50–3200 ng/ml. Although the values obtained by the radioimmunoassay were slightly higher than those determined by the liquid chromatographic method, a correlation coefficient of 0.965 ($p < 0.001$) was obtained on statistical analysis of the results (Figure 6).

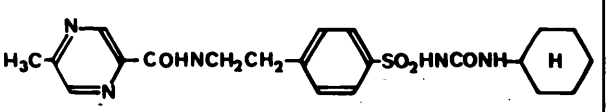
Plasma glibenclamide in patients. The radioimmunoassay was applied to the determination of glibenclamide in plasma samples obtained from patients who were treated with oral doses of 2.5 or 5.0 mg glibenclamide at 0800 h and 2.5 mg at 1700 h for at least 1 wk before blood sampling. Figure 7 shows plasma concentrations of glibenclamide in these patients. Appreciable residual concentrations of glibenclamide administered at 1700 h of the previous day were detected in the morning (0800 h) fasting plasma. Peak concentrations were observed at 1000 h and at 1900 h, 2 h after the administration of the drug.

DISCUSSION

The present paper reports on a sensitive and simple radioimmunoassay method for glibenclamide, using a specific antiserum produced by a novel method. With this procedure, it is possible to determine as little as 2.5 ng/ml of glibenclamide in plasma by using 10 μl of sample without the need for extraction. As a result of high sensitivity of the method, a small portion of plasma remaining after both insulin and glucose determinations would be sufficient for the glibenclamide assay.

FIGURE 5. Cross-reactivity of glibenclamide antiserum with some related compounds. The percentage cross-reactivity is defined as X/Y × 100, where X is the amount of unlabeled glibenclamide and Y is the amount of the test compound required to produce 50% inhibition of binding of [^{3}H]glibenclamide to the antibody.

CROSS-REACTIVITY OF GLIBENCLAMIDE ANTISERUM

COMPOUND	STRUCTURE	CROSS-REACTIVITY $\frac{X}{Y} \times 100$ %
GLIBENCLAMIDE	R_1—H R_2—H	100
4-trans-hydroxy-GLIBENCLAMIDE (M1)	R_1—OH R_2—H	0.15
3-cis-hydroxy-GLIBENCLAMIDE (M2)	R_1—H R_2—OH	0.35
GLIPIZIDE		41.7

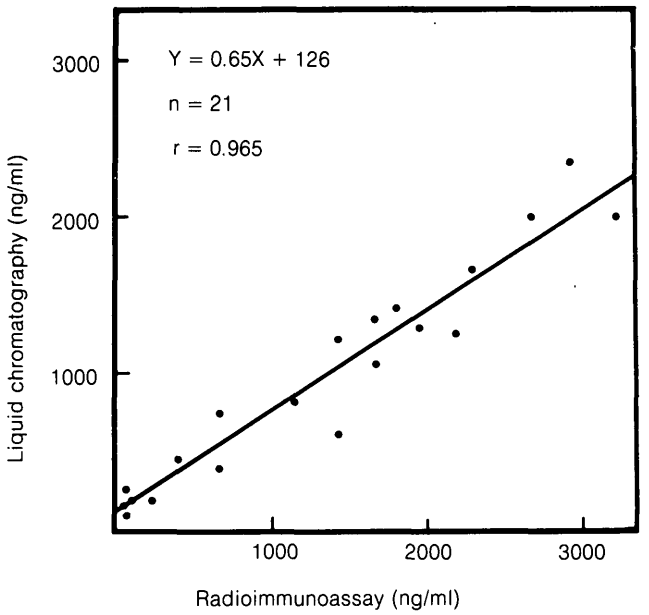


FIGURE 6. Comparison of radioimmunoassay and liquid chromatography for the determination of glibenclamide in dog plasma. Plasma samples were obtained from 7 dogs with body weight of 9 kg at various times after oral administration of 12.5 mg glibenclamide. Line was drawn from slope and the intercept values calculated by the method of least squares.

Recently, two radioimmunoassays have been reported for glibenclamide.[4,5] The antisera used in both methods exhibit considerable cross-reactivity with two major metabolites. This was because the hemisuccinate derivative of M1 was used in the preparation of immunogen or the derivative with a functional group on the cyclohexyl ring was used. To produce a highly specific antiserum, we paid considerable attention to preparing a suitable immunogen. The glibenclamide derivative, PABC, was synthesized and conjugated by diazocoupling to BSA, leaving the cyclohexyl ring intact by locating it distantly from BSA (Figure 1). This is based on two reasons: (a) two major metabolites of glibenclamide (M1 and M2) are hydroxylated derivatives at C4 or at C3 of the cyclohexyl ring,[1] and (b) antibody specificity is directed primarily against that portion of the molecule furthest from the site of conjugation to the protein carrier. As to be expected, the antiserum produced by this immunogen does not substantially cross-react with two major metabolites. The excellent correlation of the results between radioimmunoassay and liquid chromatography also confirms the specificity of this procedure.

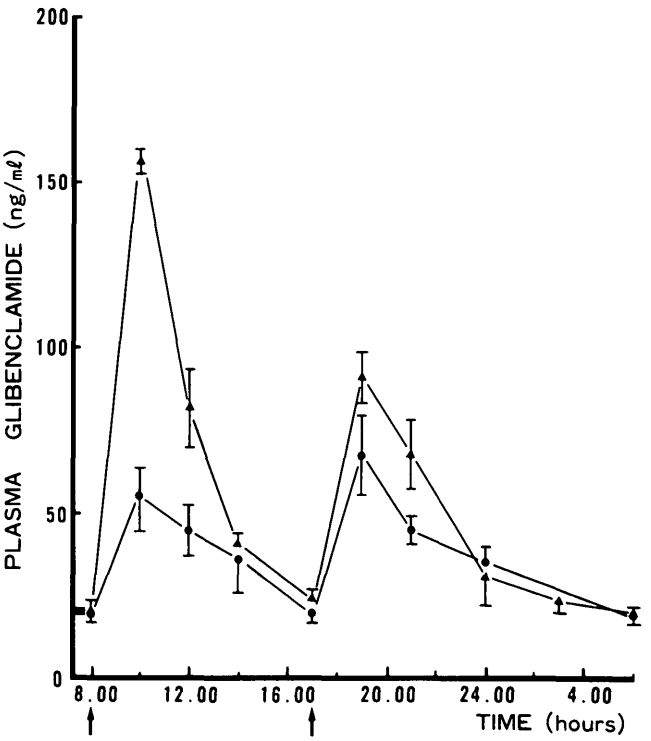
An extensive cross-reaction of glipizide with this antiserum was expected, because it has a closely related structure with glibenclamide. This cross-reaction suggests that this radioimmunoassay system would be also useful for the assay of plasma glipizide.

The plasma concentrations of glibenclamide in diabetic patients determined by our procedure were almost comparable with those reported by other investigators either in normal subjects[9] or in diabetic patients,[10] as studied by oral administration of [^{14}C]glibenclamide or by a radioimmunoassay. We consider that the residual concentrations of glibenclamide in the morning plasma determined by our radioimmunoassay is due mostly to the parent drug rather than to the metabolites or some nonspecific factors in plasma, since the antiserum virtually does not cross-react with two major metabolites (M1 and M2) and the same volume of normal human plasma was added to the standard curve tubes as to unknown tubes. The accumulation of metabolites in the morning plasma is unlikely because M1 and M2 have very high total clearances as compared with glibenclamide. The slow decline of plasma concentration observed several hours after oral administration may indicate the presence of a slowly equilibrating "deep" compartment, as suggested by Balant et al.[10]

When 100 μl of control plasma was added to the radioimmunoassay system, the percentage of [^{3}H]glibenclamide bound to antiserum was greater than that without addition of plasma, suggesting some nonspecific binding of [^{3}H]-glibenclamide to plasma proteins. Christ et al. reported that more than 99% of glibenclamide is bound to plasma proteins in vitro.[1] Our data on paper electrophoresis demonstrate that glibenclamide is bound mostly to the albumin fraction, but this binding seems to be easily dissociated in the presence of antibody with higher affinity. With the use of a smaller volume (10 μl) of plasma and the addition of the same volume of normal plasma to the series of standard curve tubes, the interference by plasma proteins could be avoided.

In conclusion, this radioimmunoassay method is suitable for the routine monitoring of glibenclamide in clinical blood samples because of its specificity, sensitivity, and simplicity. This method will make the pharmacokinetic studies easier in diabetic patients who are being treated with glibenclamide.

FIGURE 7. Plasma concentrations of glibenclamide in two groups of diabetic patients being treated for at least one week with glibenclamide. Five patients (● —— ●) received daily dose of 5 mg (2.5 mg at 0800 and 2.5 mg at 1700) and six patients (▲ —— ▲) received daily dose of 7.5 mg (5 mg at 0800 and 2.5 mg at 1700). Arrows indicate the time of drug administration. Each point represents mean and SEM.



ACKNOWLEDGMENTS

We thank Hoechst Japan and Hoechst Aktiengesellschaft for the supply of glibenclamide, [^{3}H]glibenclamide, M1, M2, and *p*-aminoethyl-benzenesulfonyl-N-cyclohexylurea. Glipizide was donated by Fujisawa Pharmaceutical. Thanks are due to Dr. Y. Sugita for help and Prof. S. Yoshida for support during this study. We are indebted to Dr. K. Fujimoto, Research Laboratory, Hoechst Japan, for the assay of glibenclamide in dog plasma by the liquid chromatography method.

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