A Radioimmunoassay for Determination of Glibenclamide and Other Sulfonylureas

Wolfgang Heptner[1,2], Mario Badian[1], Siegfried Baudner[1], Christa Hellstern[1], Robert Irmisch[1], Werner Rupp[1], Kurt Weimer[1], and Hans Wissmann[1]

Received: February 1, 1984; accepted: April 18, 1984

Abstract: An antiserum was prepared for the determination of glibenclamide and for the estimation of other commercially available sulfonylureas. Rabbits were immunized with a glibenclamide-BSA conjugate. Tritiated glibenclamide was used as the tracer. The assay was performed in the presence of 8-anilinonaphthalenesulfonic acid to displace glibenclamide bound to serum protein, and free and antibody bound tracer were separated by dextran-coated charcoal. For glibenclamide determination in serum and plasma the limit of detection was 3 ng/ml. Sensitivity calculated for the whole determination range was 102 cpm for a 10 % concentration difference. Specificity studies showed a cross-reaction of less than 0.1 % for glibenclamide metabolite M_1 and 9 % for metabolite M_2. Other sulfonylurea drugs display cross-reactivities from 0.1 % (chlorpropamide) to 190 % (gliquidone). Both intra-assay and inter-assay imprecision were below 10 %. Accuracy was established by comparison of the present method with HPLC. The assay was applied to the specific determination of glibenclamide in clinical trials and for diagnosing factitious hypoglycemia caused by sulfonylureas.

Glibenclamide (glyburide, Daonil®, HB 419, 1-[4-[2-(5-chloro-2-methoxybenzamido)ethyl]-phenylsulfonyl]-3-cyclohexylurea) is a highly potent antidiabetic agent, which is dosed at 2.5 and 5 mg per person orally. Previously, three radioimmunoassays using antisera which also react with the metabolites (1, 2, 3), a specific radioimmunoassay (4), three HPLC procedures (5, 6, 7), and a gas chromatographic method (8) have been developed for the determination of glibenclamide drug blood levels.

This paper describes a sensitive and specific radioimmunoassay for glibenclamide determination in serum and plasma. The procedure was validated according to the recommendations of the International Federation of Clinical Chemistry (9). The antiserum was also used to detect factitious hypoglycemia.

Material and Methods

Preparation of the Immunogen

The immunogen was prepared by coupling a glibenclamide derivative, 1-[4-[2-(2-aminobenzamido)ethyl]-phenylsulfonyl]-3-cyclohexylurea to bovine serum albumin (BSA) (Behringwerke, Marburg, FRG) by a dinitrophenyl bridge, according to the procedure of Tager (10). The structure of the immunogen is shown in Fig. 1. In 0.2 mol/l potassium phosphate buffer, pH 7.0, the UV spectrum of the conjugate showed maximum absorbence at 349 nm which is due to the dinitrophenyl moiety. This absorption was used to determine the coupling ratio of 25 moles glibenclamide derivative per mole BSA.

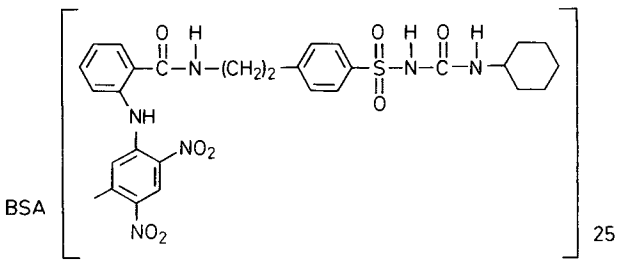


Fig. 1 Structure of the immunogen.

Production of Antiserum in Rabbits

At the start of the immunization program four New Zealand rabbits weighing 3 to 4.5 kg received 1.5 mg conjugate in 2 ml saline emulsified with 2 ml of Freund's adjuvant subcutaneously at 8 different sites in the region of the lymph nodes. On each of the following 4 days, 0.1 mg conjugate in 3 ml 0.05 % aerosil solution was administered intravenously. The same procedure was repeated 3 times at 10 day intervals. Intravenous booster injections were given over a period of 18 months. Small amounts of anitbodies were detected two months after the start of immunization. A marked increase was observed after 7 months. Thereafter every 3 weeks, approximately 60 ml blood was taken and high quality antiserum was pooled and divided in 1 ml portions, sufficient for 20 000 single determinations.

Preparation of Tracer

Tritiated glibenclamide served as the tracer and was synthesized by hydrogenating an unsaturated (cyclohex-1-enyl) precursor with tritium gas using palladium on carbon (5 %) as catalyst. The tritiated tracer was purified by TLC. The specific radioactivity of different batches was (66.6–222) $\times$ $10^7 s^{-1}$ per mg (18–60 mCi/mg). The material was stable for 3 to 8 months when stored at a concentration of 3.7 $\times$ $10^4 s^{-1}$ per ml (1 µCi/ml) assay buffer. Partially decomposed tracers can be purified by repeated cristallization or by TLC.

Other Materials

Glibenclamide sodium salt, Batch no. Op. U 002 (Hoechst AG, Frankfurt/M.) was used as standard material. All chemicals were of analytical reagent quality. Bovine serum albumin was purchased from Behringwerke, Marburg; charcoal, Norit A, from Serva, Heidelberg; Dextran T 70 from Pharmacia, Upsalla, (Sweden); 8-anilino-1-naphthalene-sulfonic acid (ANSA), from Sigma chemical company, St. Louis, (USA); scintillation cocktail 808 E, from Riedel de Häen, Hanau. Human serum and plasma were prepared from blood of healthy volunteers taken after an overnight fast. 20 ml aliquots were stored at −20°C.

[1] Hoechst AG, D-6230 Frankfurt 80
[2] Correspondence to Dr. W. Heptner, Clinical Research, H 840, Hoechst AG, P.O. Box 800320, D-6230 Frankfurt 80

0.01 mol/l phosphate buffer pH 7.4 containing 8.77 g NaCl, 1.0 g bovine serum albumin and 1.0 g NaN_3 per liter was used in all experiments.

Experiments for Establishing Optimal Assay Conditions

After oral administration of 5 mg glibenclamide tablets to healthy subjects and diabetic patients, maximal concentrations of 400 ng/ml were found in serum. Using 10 000 cpm (approximately 250 pg ^{3}H-glibenclamide) and 0.05 µl antiserum per test tube, 50 to 60 % tracer binding was achieved. In the routine assays, 50 µl unknown serum was used, permitting the determination up to 200 ng/ml. Above this concentration smaller amounts of serum have to be used. Between 10 and 50 µl normal human serum there is no difference in glibenclamide binding to the antibody, while higher serum additions caused a remarkable decrease in binding.

The separation of antibody-bound and unbound glibenclamide was achieved by the addition of dextran-coated charcoal suspension. 2.5 g charcoal and 0.25 g dextran were suspended in 100 ml assay buffer with continuous stirring at 4 °C. Constant adsorption is observed for incubation times of between 10 and 30 min.

The kinetics of tracer binding to the antibody were studied at room temperature and at 4°C. An increase in tracer binding was observed for up to 17 hours when incubated at 4°C, while at room temperature the binding was lower. These experiments favor incubation periods of between 17 and 24 hours at 4°C.

Influence of 8-Anilino-naphthalene-sulfonic Acid (ANSA) on Binding of Tracer to the Antibody

Glibenclamide is more than 99 % bound to serum proteins (11). It is well known that high protein binding may influence drug concentration measured by RIA, as demonstrated with the thyroid hormone T_4 (12). ANSA was shown to displace T_4 from serum proteins. Therefore, the influence of ANSA on the binding of glibenclamide tracer to the antibody was investigated. The tracer-antibody binding increases with increasing concentrations of ANSA. In the routine assay 600 µg ANSA per test tube were used.

Routine Assay Procedure

Assay buffer was used to prepare working dilutions of tracer (100 000–150 000 cpm per ml, counting efficacy of the well-type β-counter: 50 %), antiserum (0.5 µl/ml), standards (1.5, 3.1, 6.2, 12.5, 25.0, 50.0, 100.0 ng/ml) and interassay controls (3.0, 10.0, 50.0 ng/ml). Details of the procedure are given in Table I. Determinations were carried out in triplicate in polystyrene tubes (12 × 55 mm). After incubating overnight, 0.5 ml dextran-coated charcoal was added for the absorption of free glibenclamide. The tubes were then mixed, left to stand for 10 min, and finally centrifuged at 2500 g and 4°C for 10 min. The radioactivity of the supernatant was determined in a well-type β-counter. The concentrations were read from the standard curve using a non-linear spline approximation (13).

Results

Specificity

In healthy subjects two metabolites (Table II) of glibenclamide were identified. M_1 (4-*trans*-hydroxy-glibenclamide) was present in blood and urine, while M_2 (3-*cis*-hydroxy-glibenclamide) could only be detected in the urine (14).

Assay specificity was established by incubating increasing concentrations of the drug and metabolites with the antibody. The results of these experiments are summarized in Fig. 2. By

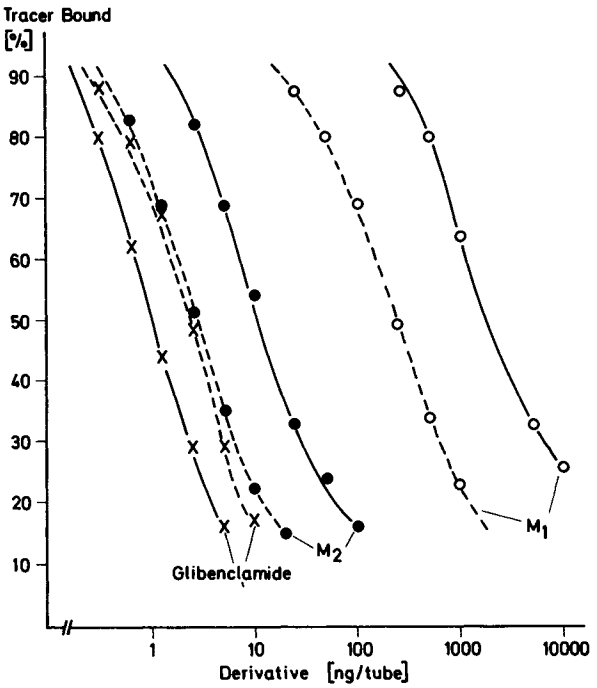


Fig. 2 Standard curve for glibenclamide determination x—x and cross-reaction of the metabolites M_1 and M_2. For formulas see Table II. Standard assay conditions were used. —— curves ANSA added, ---- curves without ANSA. Each value is the mean of 3 measurements. Cross-reactivity of the metabolites is <0.1 % for M_1 and 9 % for M_2 in the presence of ANSA.

Table I. Assay protocol for determination of glibenclamide by RIA (all volumes are given in ml/tubes)

	ANSA solution	Human serum	Buffer	Standard solution	Tracer solution	Antiserum solution
Total counts	0.1	0.05	0.7	–	0.1	–
Absorption binding	0.1	0.05	0.2	–	0.1	–
O-binding	0.1	0.05	0.1	–	0.1	0.1
Standard	0.1	0.05	-	0.1	0.1	0.1
Controls	0.1	0.05	0.1 control solutions	–	0.1	0.1
Unknowns	0.1	0.05	0.1	–	0.1	0.1

Table II. Cross-reaction of sulfonylureas in the glibenclamide RIA-system. Cross-reactivity and determination range were assessed by a standard curve for each compound. The degree of cross-reactivity was calculated by the concentrations which can achieve a 50 % displacement of the tracer.

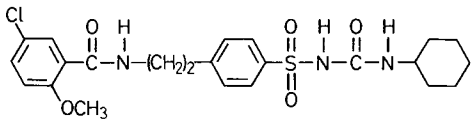
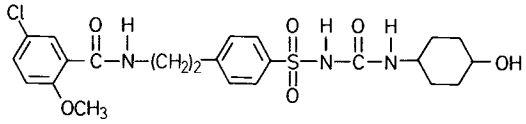
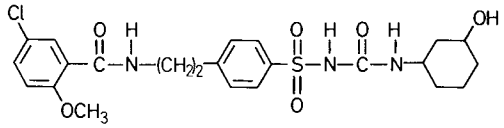
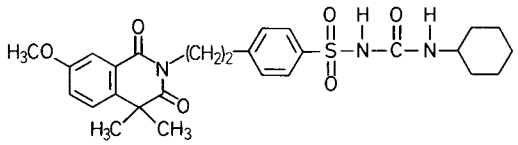
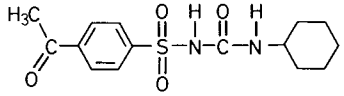
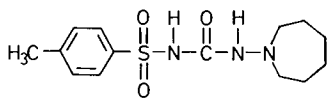
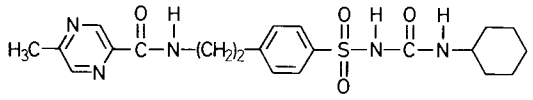
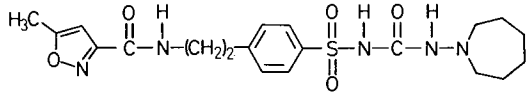
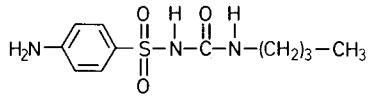
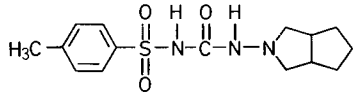
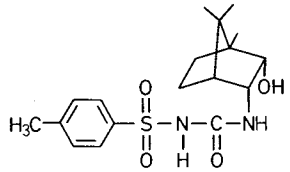
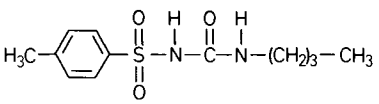
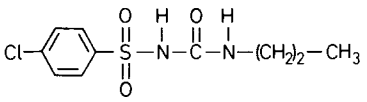
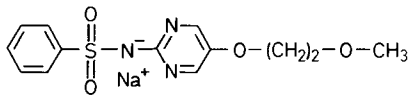
Generic Name	Trade Name		Cross-Reaction (%)	Determination Range (Glibenclamide Equivalents in ng/ml)
Glibenclamide Glyburide	Euglucon 5 Daonil Euglucon N Diabeta		100	2–200
Glibenclamide Metabolite M_1			0.1	500–>10000
Glibenclamide Metabolite M_2			9.0	30–2000
Gliquidone	Glurenorm		190	2–100
Acetohexamide	Dimelor Dymelor Dimelin		180	2–100
Tolazamide	Tolinase Norglycin		169	2–200
Glipizide	Glibenese Glucotrol Minidiab		143	2–100
Glisoxepide	Pro-Diaban		68	3–500
Carbutamide	Nadisan Invenol Glucidoral		44	3–400
Gliclazide	Diamicron		37	5–500
Glibornuride	Glutril		4	25–>10000

Table II. (continued)

Tolbutamide	Orinase Rastinon Artosin Dolipol		2	5–5000
Chlorpropamide	Diabenese Diabetoral Chloronase		0.1	50–10000
Glymidine	Redul Glyconormal		0	

comparing the concentrations which achieved a 50 % displacement of the tracer, the cross-reactivity of glibenclamide metabolite M_1 is less than 0.1 %, and that of M_2 is 9 %. In the absence of ANSA the values for cross-reactivity are shifted to 1 % for M_1 and to 89 % for M_2.

The influence of commercially available antidiabetic sulfonylureas was investigated in analogue binding studies. For the compounds tested, cross-reactivity and determination range are listed in Table II. The highest cross-reaction was shown for gliquidone (190 %) and the lowest was determined for chlorpropamide (0.1 %). The sulfonamide glimidine had no influence on tracer displacement.

Detection Limit

The mean and standard deviation of the radioactivity of blank samples were determined for each standard curve in triplicate. The concentration corresponding to the 3 ×SD level was taken as the detection limit, with values ranging from 1 to 3 ng/ml. The assay detection limit was set uniformly at 3 ng/ml.

Slope of the Calibration Curve

The slope of the calibration curve as a measure of assay sensitivity was determined by the extent of change in the analytical response relative to the change in glibenclamide concentration. An estimate of sensitivity is the difference of radioactivity bound (cpm) caused by a 10 % change in concentration. The calculations were performed for 3 different segments, i.e. for the endpoints and for the linear segment of the calibration curve. In the linear part it was 122 cpm, and in the initial and final segments the value decreased to 60 cpm. The overall estimate was 102 cpm for a 10 % difference in concentration.

Reproducibility

Intra-assay imprecision was tested by recovery experiments. Glibenclamide was added to human serum at concentrations ranging from 5 to 500 ng/ml. The resulting samples were divided into 10 aliquots and glibenclamide was measured by RIA. For the concentrations 300 and 500 ng/ml, a 20 µl serum aliquot was used. The results are given in Fig. 3. The coefficients of variation ranged from 2.6 to 9.8 %.

To assess the inter-assay imprecision, 3 different control solutions were prepared by dissolving weighted amounts of glibenclamide in assay buffer. In 10 replicates the interassay imprecision was 12 % at 3.1 ng/ml, 5.7 % at 10 ng/ml and 4.1 % at 50 ng/ml.

Linearity

Linearity was tested using the results of the recovery experiments. A log-log plot of these data is given in Fig. 3. The calculation of the log-log-regression resulted in

$$\ln C_{measured} = (0.02 \pm 0.02) + (0.994 \pm 0.006) \ln c_{added}$$

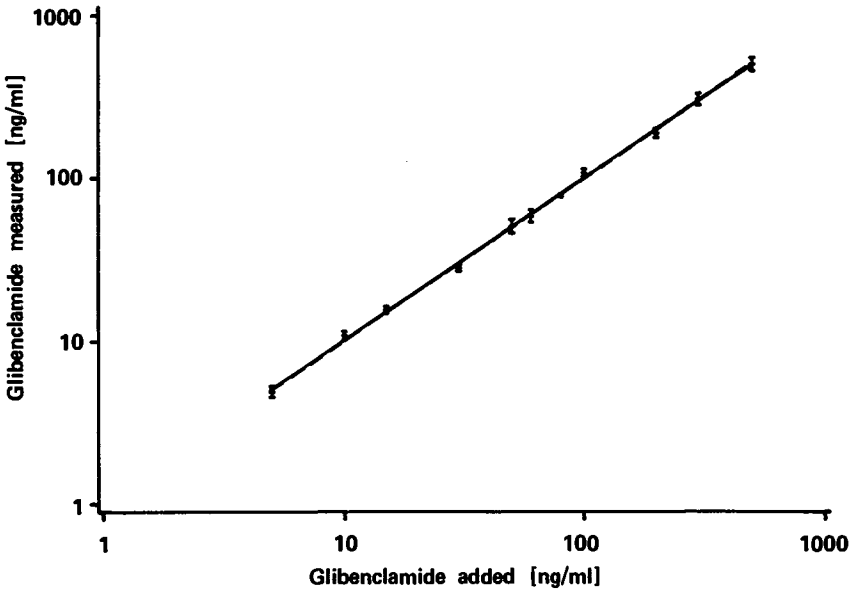


Fig. 3 Log-log plot of measured concentrations in spiked samples. Each point is a mean of 10 determinations ± 1 SD.

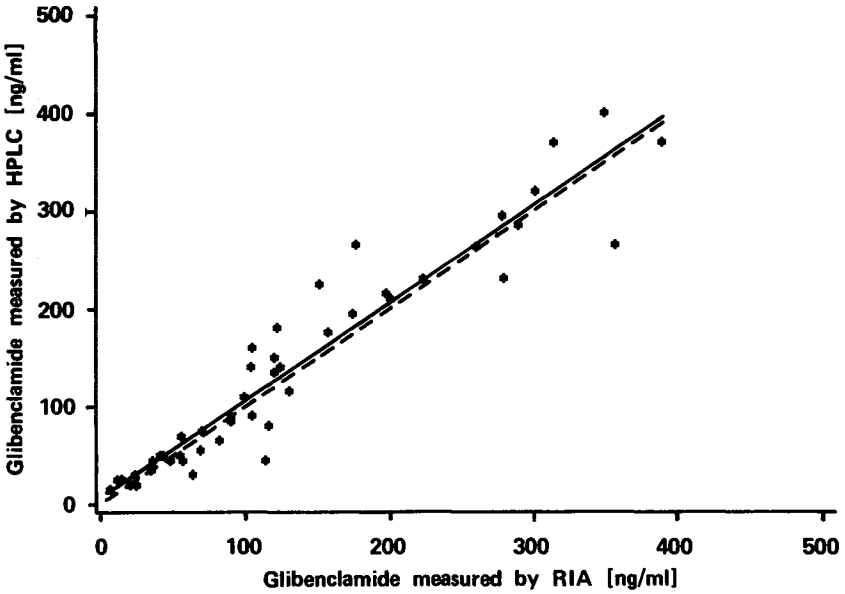


Fig. 4 Method comparison of RIA and HPLC for the determination of glibenclamide. Scatter plot of parallel determinations in human serum (n = 47).

Both intercept and slope were not significantly different from 0 or 1 respectively, thus confirming linearity. In addition linearity was shown by Fisher's test for lack of fit (15). The F value was less than 1.

Accuracy

Accuracy was determined by a recovery experiment and by parallel determinations with RIA and HPLC in true samples from a human trial. According to the recovery experiment (Fig. 3), the relative bias values (difference between the mean of measured values and added concentrations) ranged from −5.7 to +8.0%.

After oral administration of 2.5 mg glibenclamide sodium salt to eight healthy subjects blood was taken after 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 hours. Glibenclamide was measured by HPLC (5) and RIA. The results are shown in the scatter plot (Fig. 4). Pairs with one value below the detection limit were omitted. The linear regression calculation resulted in

$$C_{HPLC} = (6.5 \pm 7.9) + (0.998 \pm 0.047)\, c_{RIA} \quad (n = 47).$$

Both intercept and slope are not significantly different from 0 and 1 respectively.

In addition, a time plot of the relative difference between HPLC and RIA is shown in Fig. 5. The relative difference was calculated as difference between values expressed as a percentage of the mean of both values (9)

$$\text{relative difference} = 100\, \frac{c_{HPLC} - c_{RIA}}{(c_{HPLC} + c_{RIA})/2}\,.$$

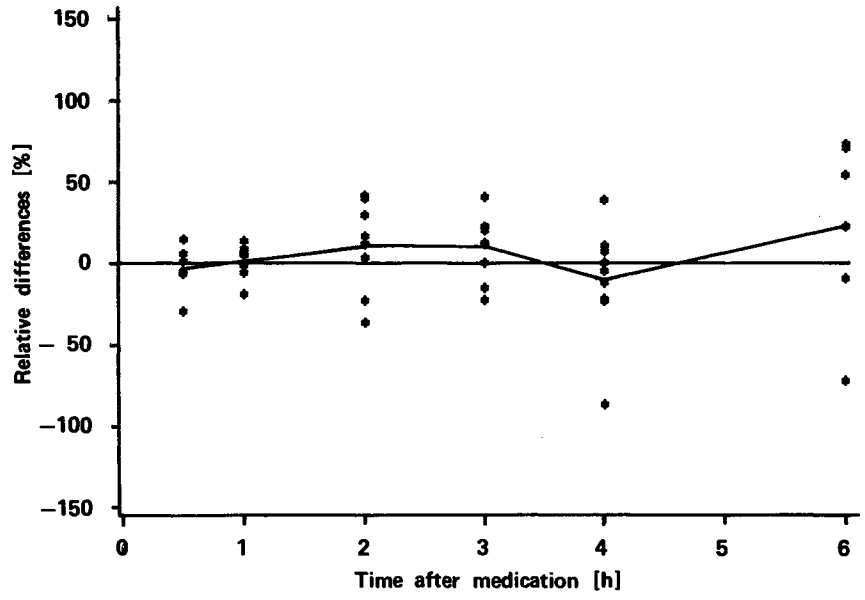


Fig. 5 Comparison of RIA and HPLC in human serum (n = 47). The relative differences between HPLC and RIA are plotted versus time after medication. Positive differences correspond to $c_{HPLC} > c_{RIA}$.

The plot shows that there is no time-dependent trend in the estimation of the methods which may result from metabolite interference. The range of relative differences increased slightly with time after drug administration.

Discussion

After a long immunization period the selected immunogen yielded an antiserum discriminating between glibenclamide and the metabolite M_1. The cross-reactivity of the metabolite M_2 (9 %) is of no importance since this compound was only detected in urine and not in human serum (14). In presence of ANSA there is a higher degree of glibenclamide binding to the antibody, and the specificity was improved markedly. This can be explained by a partial displacement of the protein-bound portion (99 %) of glibenclamide by ANSA. During the development of a radioimmunoassay for tyroxine a similar finding was observed and described by Chopra (12). ANSA displaced T_4 from tyroxine binding globulin. In contrast to the marked specificity of the antiserum for glibenclamide in the presence of M_1, there is a high cross-reactivity of more than 100 % with some other sulfonylureas (gliquidone, glipizide, acetohexamide, tolazamide) with completely different structures. The common characteristic of the compounds with high cross-reactivity is an unsubstituted cyclohexyl ring (except tolazamide) directly connected to the urea group. If the substituent at the urea nitrogen, remote from the sulfonyl group, is changed, the cross-reaction is diminished to varying degrees. The structure of the part of the molecule bound to BSA has little influence on specificity. As may be expected the sulfonamide glimidine showed no cross-reactivity.

The whole procedure, i.e. quality of reagents, antiserum, tracer and the constancy of counting is controlled by measuring total counts, nonspecific binding and the concentration of 3 control samples. No interference has been observed in blank samples from healthy subjects so far. No influence of other drugs except sulfonylureas has been observed in patients under multiple drug treatment. The method is only suitable for human and not animal serum as the shapes of the calibration curves are different.

The method has been validated with respect to specificity, sensitivity, detection limit and reproducibility. Accuracy and linearity were demonstrated by recovery experiment. Statistical calculations demonstrated a strong correlation between the glibenclamide concentrations added and RIA results. There is good agreement between both HPLC and RIA determinations in human serum. The cross-reactivity of commercially available sulfonylureas permits the diagnosis of factitious hypoglycemia caused by sulfonylureas (16).

Kawashima et al. (4) used a different drug conjugate to prepare an antiserum as described in the present paper. Assay characteristics of the two RIA-procedures are similar. However, the RIA described by Kawashima measures remarkably higher glibenclamide concentrations than a reference liquid chromatography method. In comparison to physico-chemical procedures the present RIA is attractive, as processing a large number of samples is simple and fast and the volumes of serum required are small.

References

(1) Hrstka, V. E., Schmidt, F. H. (1973) Abstr. 88. 8. Kongreß der Deutschen Diabetes-Gesellschaft, München.
(2) Głogner, P., Burmeister, P., Heni, N. (1973) Drug Res. 51, 352.
(3) Royer, M. E., Ko, H., Evans, J. S. (1976) Anal. Let. 9, 629–640.
(4) Kawashima, K., Kuzuya, T., Matsuda, A. (1979) Diabetes 28, 221–226.
(5) Uihlein, M., Sistovaris, N. (1982) J. Chromatogr. 227, 93–101.
(6) Lindner, G., Herberts, L., Reinauer, H. (1980) Lab. Med. 4, 34–38.
(7) Rogers, H. J., Spector, R. G., Morrison, P. J., Bradbrook, I. D. (1982) Diabetologia 23, 37–40.
(8) Castoldi, D., Tofanetti, O. (1979) Clin. Chim. Acta 93, 195–198.
(9) Büttner, J., Borth, R., Boutwell, J. H., Broughton, P. M. G., Bowyer, R. C. (1976) Clin. Chemist (N.Y.) 22, 1922–1932.
(10) Tager, H. S. (1976) Anal. Biochem. 71, 367–375.
(11) Hajdù, P., Kohler, K. F., Schmidt, F. H., Spingler, H. (1969) Drug Res. 19, 1381–1386.
(12) Chopra, I. J. (1972) J. Clin. Endocrinol. 34, 6, 938–947.
(13) Stoer, J. (1976) Springer Verlag, Berlin, Heidelberg, New York, 76–100.
(14) Rupp, W., Christ, O., Heptner, W. (1969) Drug Res. 19, 1428–1434.
(15) Draper, N. R., Smith, H. (1966) John Wiley and Sons New York, Chichester, 33–40.
(16) Heptner, W., Baudner, S., Hellstern, Ch., Ökonomopulos, R., (1983) II. World Conference on Clinical Pharmacology and Therapeutics, Washington, Jul. 31–Aug. 5.