expressed as micrograms per milliliter, *versus* time in hours showed that the disappearance of I from plasma was biphasic. The half-life for the terminal phase, as estimated graphically from the data between 6 and 24 hr after drug administration, was 6.2 hr. The combined results from these investigations showed that the GLC method could be used for: *(a)* evaluating the pharmacokinetics, *(b)* evaluating drug availability from various dosage formulations, and *(c)* selecting an optimum dosage regimen for I administration to humans.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 23, 1973, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication November 26, 1973.

The authors thank Dr. C. D. Brooks for conducting the clinical portion of the study and Mr. W. F. Liggett for technical assistance.

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Interactions of Sulfonylureas with Plasma Proteins

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Abstract □ The binding of sulfonylureas to plasma proteins was studied by a fluorescence method, using 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV) as fluorescence probes. Chlorpropamide, acetohexamide, and tolbutamide competed with IV but not with III in binding to bovine serum albumin. In the cases of glipizide (I) and glyburide (glibenclamide) (II), however, competitive binding between these drugs and III was observed. In contrast to the decrease in intensity, fluorescence enhancement of IV by I and by II was found in human serum albumin and in both human and bovine serum albumins, respectively. The fluorescence of warfarin in bovine serum albumin solution was increased by the addition of I. This may indicate the formation of ternary complexes between these drugs and the probe-protein complex; as a result, these drugs may enhance the protein binding of the probe or drugs that compete with the probe for the same binding sites. In comparison with other sulfonylureas, I and II bind to different but closely located sites on the protein with greater affinities.

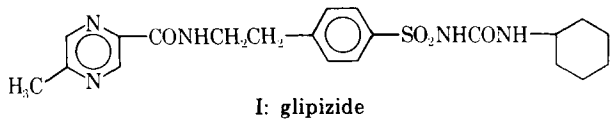
Keyphrases □ Sulfonylureas—binding to plasma proteins, fluorescence probe technique □ Plasma proteins—binding of sulfonylureas, fluorescence probe technique □ Binding, sulfonylureas to plasma proteins—studied using fluorescence probe technique □ Fluorescence probe technique—used to study sulfonylurea binding to plasma proteins

Binding of drugs to plasma proteins has long been known as an important factor in drug availability, drug efficacy, and drug transport (1). It has been shown (2–4) that the unbound drug in plasma is considered to account for the drug's pharmacological activity. In other words, the correct therapeutic plasma level is not the total drug concentration in plasma but the unbound portion of the drug. The binding occurs mainly in the albumin fraction of the plasma and is reversible (5). Brand *et al.* (6) showed that drugs having low affinities in binding to albumin are at least partially dissociated from their protein binding sites by drugs with higher affinities and, to a lesser extent, by those with equal or lesser affinities. This is true provided the binding of these drugs occurs at the common or adjacent sites on the protein. The dissociation of a drug from its binding sites by a suitable competitor may cause an enhancement of activity of that drug and can otherwise alter its metabolism. The binding affinity of one drug may also be enhanced by the presence of another drug due to drug interactions and drug–protein complexations.

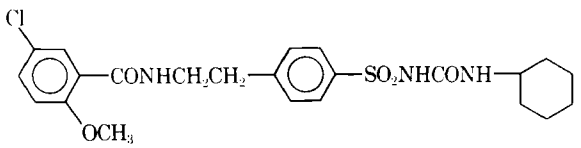
Chlorpropamide, acetohexamide, and tolbutamide are commonly used sulfonylureas. Protein binding of these drugs has been tentatively studied using such techniques as ultracentrifugation (7), equilibrium dialysis (8–11), ultrafiltration (9), and electrophoresis (9, 12). In the study reported here, a fluorescence probe technique (13) was used because this method provides an excellent model to show the competitive binding character between drugs and probe or possibly other drugs in binding to plasma protein.

Recently, two new hypoglycemic agents, glipizide (I) and glyburide (glibenclamide) (II), were reported (14). Metabolism and kinetics studies (14–16) showed that these drugs are extensively bound to plasma proteins and showed a limited volume of distribution. Because of the structural differences, the binding of glipizide and glyburide to plasma protein may differ from those of other sulfonylureas. Therefore, to understand further the pharmacological activities of these drugs, quantitative studies of their protein binding are needed. In this respect, the binding of glipizide and glyburide to different proteins was also studied using the fluorescence probe technique.

Two probes, 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV), were used in this study. The synthesis and usage of III as a fluorescence probe were previously studied in this laboratory (17).



I: glipizide



II: glyburide

EXPERIMENTAL

Materials—Tolbutamide[1], chlorpropamide[2], acetohexamide[3], glipizide[4], glyburide[5], sodium warfarin[6], and 1-anilinonaphthalene-8-sulfonic acid[7] were obtained from commercial sources. The sodium salt of III was prepared in this laboratory (17). All other chemicals were reagent grade or of special purity.

Apparatus and Methods—Fluorescence measurements were made with a spectrophotofluorometer[8] equipped with a 150-w xenon lamp and a 1P21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings. Fluorescence spectra were recorded with an *X-Y* recorder[9]. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 3 and 4 mm, respectively.

Fluorescence titrations of protein solutions with probes in the absence and presence of sulfonylureas were carried out manually with microsyringes[10]. Two milliliters of each protein solution was titrated with successive additions of 2 μl of 1×10^{-3} *M* probe solution dissolved in methanol. After each titration, the fluorescence intensity was recorded as a function of probe concentration. The excitation and emission wavelengths were taken to be 420 and 485 nm for III and 375 and 475 nm for IV, respectively. The protein solutions were prepared in pH 7.4 phosphate buffer (0.05 *M*). A molecular weight of 69,000 was used for bovine serum albumin. Because of their low solubility in water and in methanol, all sulfonylureas studied were dissolved in 0.1 *N* NaOH solution. The pH and the ionic strength of all solutions were controlled at constant values by use of 0.1 *N* NaOH solution. The final pH was 7.45. The temperature of all measurements was maintained at 25 ± 0.1°.

The binding of probes and sulfonylureas to 1.38×10^{-6} *M* bovine serum albumin was determined by the methods previously used in these laboratories (18, 19). To calculate the fractions of free and bound probe concentrations, fluorescence data of the probe at a high protein concentration, which provides excess protein binding sites, are required. In this study, the fluorescence titrations were carried out at several protein concentrations, and the fluorescence data of the probe at high protein concentration were obtained by an extrapolation method. The binding parameters of the probe to the protein were calculated using the Scatchard (20) equation. Fluorescence titrations of 1.38×10^{-6} *M* bovine serum albumin with probes were also carried out in the presence of sulfonylureas. The competitive binding of these drugs to the protein were determined using the Klotz *et al.* (21) equation. All fluorescence data were the result of six separate determinations.

RESULTS AND DISCUSSION

Compounds III and IV were used as fluorescence probes in the study of the interaction of sulfonylureas to plasma proteins. The binding of III to bovine serum albumin has been studied using a fluorescence method. The Scatchard plot and the binding parameters are shown in Fig. 1 and Table I, respectively. It was found that III binds to bovine serum albumin in an approximate 1:1 molar ratio. A previous report (17) showed that the carboxylate group of III interacts with a positively charged residue on the protein molecules. Hydrophobic interactions between the protein and the compound are also expected. The fluorescence changes of III by solvents and the protein environment (17) are very similar to those of IV (22, 23) and may be interpreted as a result of the charge transfer between the probe and the protein molecules. The binding constants of these two probes are approximately the same; however, the number of binding sites for the binding of IV to bovine serum albumin is three.

Interactions of glipizide and glyburide to bovine serum albumin were studied using III as the indicating probe. It was found that these drugs compete with the probe for the same binding sites on the protein. The results are shown in Fig. 1 and Table I. Other

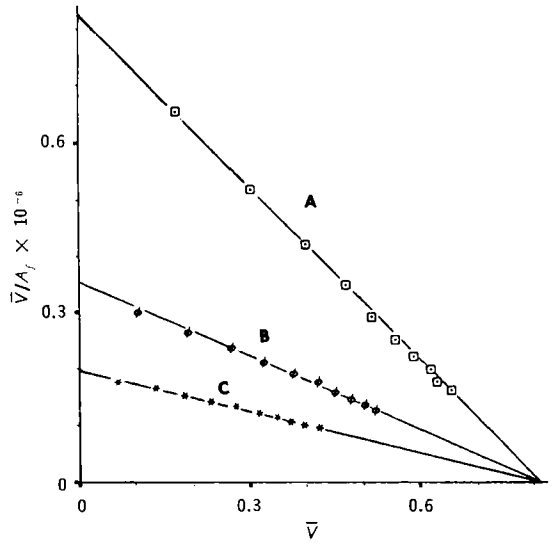


Figure 1—*Scatchard plots for the binding of III to bovine serum albumin. Key: A, in the absence of drug; B, in the presence of* 2×10^{-5} M *glipizide; and C, in the presence of* 1×10^{-5} M *glyburide.* $\bar{V}$ *is the number of moles of bound probe per mole of protein, and* A_f *is the free probe concentration.*

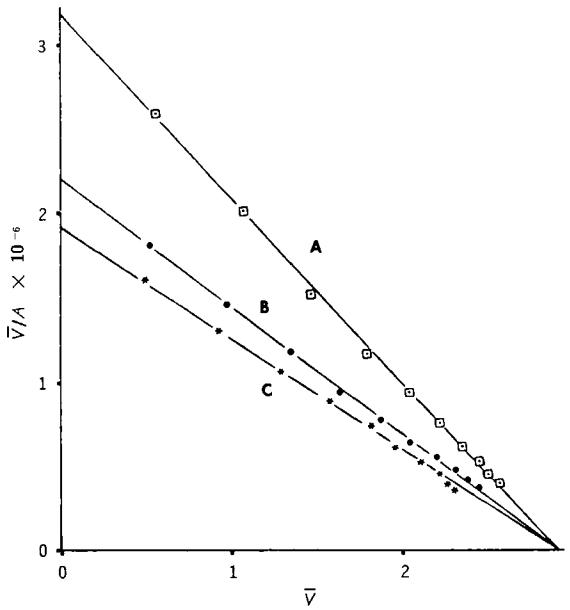


Figure 2—*Scatchard plots for the binding of the probe IV to bovine serum albumin (*1.38×10^{-6} M*). Key: A, in the absence of drug; B, in the presence of* 2×10^{-5} M *glipizide; and C, in the presence of* 2×10^{-5} M *acetohexamide.* $\bar{V}$ *is the number of moles of bound probe per mole of protein, and* A_f *is the free probe concentration.*

[1] Upjohn Co., Lot No. 342AE.
[2] Pfizer Inc., Lot No. 19559.
[3] Eli Lilly and Co., Lot No. QA 106A.
[4] Instituto Carlo Erba Per Ricerche Therapeutiche, Italy, Lot No. K4024.
[5] Upjohn Co., Lot No. 8707-KGW-2D.
[6] Abbott Laboratories, Chicago, Ill., Lot No. 833-1521.
[7] Aldrich.
[8] Aminco Bowman, American Instrument Co., Silver Spring, Md.
[9] Omnigraphic, Houston Instrument, Bellaire, Tex.
[10] Hamilton.

Table I—Binding Constants of Sulfonylureas to Bovine Serum Albumin[a]

Compound	$A(n = 2.90)$	$B(n = 0.81)$
IV	1.10×10^6	—
III	—	1.02×10^6
Chlorpropamide	2.02×10^4	—
Acetohexamide	3.76×10^4	—
Tolbutamide	9.04×10^4	—
Glipizide	2.45×10^4	6.88×10^4
Glyburide	—	3.26×10^5

[a] A = determined by the use of IV as the probe, B = determined by the use of III as the probe, and n = number of binding sites for the probes.

sulfonylureas such as chlorpropamide, acetohexamide, and tolbutamide did not affect the fluorescence or the binding of III to bovine serum albumin. When using IV as the probe, however, competitive binding between the probe and chlorpropamide, acetohexamide, and tolbutamide was observed. The results, as indicated by the Scatchard plots, are shown in Figs. 2 and 3. The binding constants, calculated from the Klotz *et al.* (21) equation, are listed in Table I. Of the three, tolbutamide and chlorpropamide have the highest and the lowest binding affinities to bovine serum albumin, respectively. The results also show that III and IV are bound to bovine serum albumin at different sites.

The binding mechanism for glipizide and glyburide to serum albumins is slightly different from that of other sulfonylureas. The fluorescence change of the protein-IV mixtures by the addition of glipizide and of glyburide is shown in Table II. Displacement of the probe by these two drugs is clearly shown in rat, rabbit, horse, and methylated bovine albumins and bovine α-globulin. However, the fluorescence change is different when the protein is human or bovine serum albumin, two proteins known to be very similar to each other. Figure 4 shows the effect on the fluorescence emission of the probe-bovine serum albumin mixture by glipizide and glyburide. An increase of the intensity accompanied by a blue shift of 10 nm was observed in the case of glyburide, whereas the addition of glipizide resulted in quenching of the fluorescence intensity. In the probe-human serum albumin system (Fig. 5), the fluorescence intensity was enhanced by both glipizide and glyburide and the emission maximum was also shifted toward the blue. Furthermore, neither glipizide nor glyburide showed fluorescence in protein solutions, nor is the fluorescence of IV changed by the addition of these drugs. Glyburide affects the binding of the probe to bovine serum albumin in a similar fashion. Binding of glipizide to bovine serum albumin, however, is somewhat different from that of glyburide. The cause of this effect is not clear; it is probably due to a slight difference in structure of glipizide and glyburide or to the structural and/or conformational differences of human and bovine serum albumins.

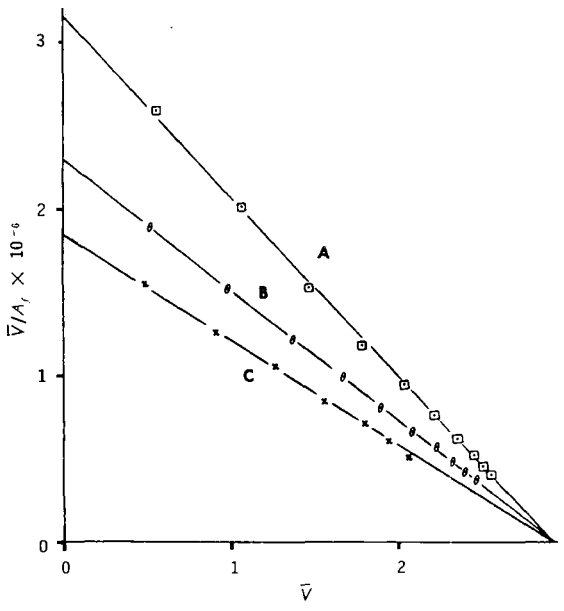


Figure 3—*Scatchard plots for the binding of the probe IV to bovine serum albumin (1.38 × 10⁻⁶ M). Key: A, in the absence of drug; B, in the presence of 2 × 10⁻⁵ M chlorpropamide; and C, in the presence of 1 × 10⁻⁵ M tolbutamide. $\bar{V}$ is the number of moles of bound probe per mole of protein, and A_f is the free probe concentration.*

Table II—Effect of Fluorescence Intensity of IV–Anilino Protein Mixtures by the Addition of Glyburide and Glipizide[a]

Protein	Addition of Glyburide	Addition of Glipizide
Bovine serum albumin	+	−
Bovine serum albumin V	+	−
Bovine serum albumin, methylated	−	−
Human serum albumin	+	+
Human serum albumin V	+	+
Horse albumin V	−	−
Rat albumin	−	−
Rabbit albumin	−	−
Bovine α-globulin	−	−
Bovine β,γ-globulin	No change	No change

[a] + = increase and − = decrease in intensity.

Drugs that are competitively interacting with IV for the same protein binding sites may also be affected by glipizide and glyburide. For example, the effect on the fluorescence of warfarin in the presence of bovine serum albumin by glipizide and glyburide is shown in Fig. 6. Warfarin has been shown (4) to compete with IV for the same sites in binding to bovine serum albumin. As shown in Fig. 6, the fluorescence effect of warfarin in bovine serum albumin solution by these drugs is very similar to that shown in Fig. 4. The pharmacological activity of a drug is greatly affected by its protein binding. The binding enhancement caused by another drug such as in the warfarin-glyburide system of bovine serum albumin may therefore reduce its drug activity.

Glipizide and glyburide do compete with IV in binding to methylated bovine serum albumin (Table II). This suggests that ionic binding, probably by means of the —CONH— groups in these drugs, also plays an important role and may be primarily responsible for the difference from other sulfonylureas in binding to plasma protein. In methylated bovine serum albumin, these methyl groups not only increase the hydrophobicity of the protein but also reduce the capacity of hydrogen bond formation between the drug and the protein. As a result, the reducing hydrogen bonding ability of bovine serum albumin alters drug interactions with the probe.

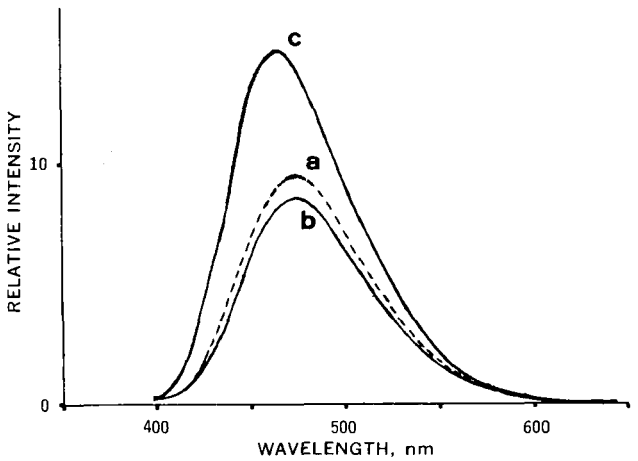


Figure 4—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁶ M IV and 0.1 mg/ml bovine serum albumin (excitation 375 nm, pH 7.45). Key: a, in the absence of drug; b, in the presence of 2 × 10⁻⁵ M glipizide; and c, in the presence of 1 × 10⁻⁵ M glyburide.*

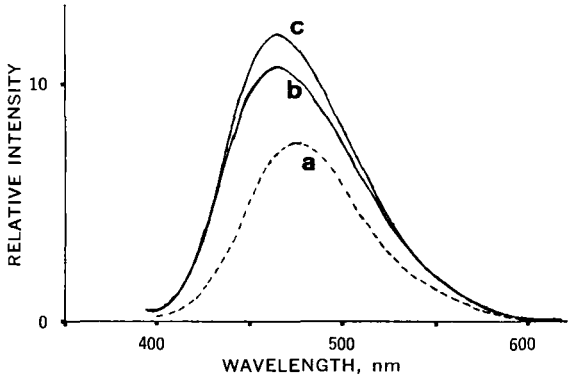


Figure 5—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁶ M IV and 0.1 mg/ml human serum albumin (excitation 375 nm, pH 7.45). Key: a, in the absence of drug; b, in the presence of 2 × 10⁻⁵ M glipizide; and c, in the presence of 1 × 10⁻⁵ M glyburide.*

SUMMARY

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV) were used as fluorescence probes in the study of the interactions of sulfonylureas to plasma proteins. The results show that these two probes may bind to different but closely located sites on the protein. Among the sulfonylureas reported in this study, chlorpropamide, acetohexamide, and tolbutamide compete with IV but not with III for the binding sites on bovine serum albumin. On the other hand, glipizide and glyburide were found to compete with III in binding to bovine serum albumin. In contrast to the decrease in fluorescence intensity, fluorescence enhancement of IV by glipizide and by glyburide was found in human serum albumin and in both human and bovine serum albumins, respectively. These results indicate that glipizide and glyburide may bind to different but closely located sites on the protein as compared to the binding of other sulfonylureas. Glipizide and glyburide may simultaneously bind to the protein and the drug bound to closely located sites on the protein to form ternary complexes and, therefore, enhance the binding of the drug to the protein.

The binding constants of these sulfonylureas to bovine serum albumin were calculated; the results show that glyburide has the highest affinity to the protein.

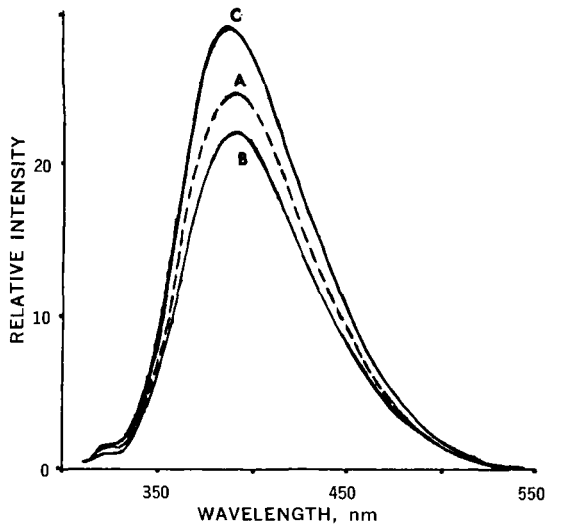


Figure 6—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁵ M sodium warfarin and 0.1 mg/ml bovine serum albumin (excitation 315 nm, pH 7.45). Key: A, in the absence of drug; B, in the presence of 2 × 10⁻⁵ M glipizide; and C, in the presence of 2 × 10⁻⁵ M glyburide.*

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ACKNOWLEDGMENTS AND ADDRESSES

Received September 24, 1973, from the *School of Pharmacy, West Virginia University, Morgantown, WV 26506*

Accepted for publication December 12, 1973.

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