GLC Determination of *dl*-2-(2-Fluoro-4-biphenylyl)propionic Acid (Flurbiprofen) in Plasma

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Abstract □ To evaluate the pharmacokinetics and drug availability from various dosage formulations, a method for the determination of *dl*-2-(2-fluoro-4-biphenylyl)propionic acid (flurbiprofen) in human plasma was required. A sensitive and specific procedure, based on *(a)* benzene extraction of the acidified specimen, *(b)* TLC of the benzene extract residue, *(c)* formation of the pentafluorobenzyl esters of the materials eluted from the thin-layer chromatogram, and *(d)* quantification of the pentafluorobenzyl esters by GLC, utilizing electron-capture detection, was developed. The lower level of assay detection sensitivity for measurement of flurbiprofen in plasma is 0.05 μg/ml. Statistical analyses indicated an average recovery of 97.1% with a standard deviation of ±9.2%. IR and mass spectrometric analyses, in conjunction with GLC, confirmed the specificity of the method for the intact drug. The procedure was successfully applied to drug absorption studies in humans.

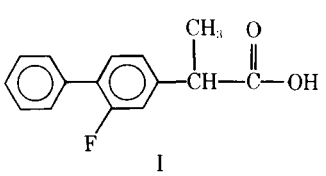
Keyphrases □ *dl*-2-(2-Fluoro-4-biphenylyl)propionic acid (flurbiprofen)—GLC analysis in plasma □ Flurbiprofen [*dl*-2-(2-fluoro-4-biphenylyl)propionic acid]—GLC analysis in plasma □ GLC—analysis, flurbiprofen in plasma

In a continuing search for nonsteroidal anti-inflammatory drugs, flurbiprofen [*dl*-2-(2-fluoro-4-biphenylyl)propionic acid] (I) was observed (1) to be one of the most potent members of a series of substituted phenylalkanoic acids, based upon a wide range of anti-inflammatory, analgesic, and antipyretic tests in various animal species. Double-blind crossover clinical trials showed I to be well tolerated, nontoxic, and orally effective in the treatment of rheumatoid arthritis in humans (2, 3).

To study the absorption, metabolism, and excretion of this agent in animals and humans at relatively low doses (*i.e.*, 10 mg), a highly sensitive and specific method for drug analysis in plasma was needed. Since earlier investigations (4) showed GLC to be a generally useful tool for measuring structurally related compounds in biological matrixes, a method utilizing GLC in conjunction with electron-capture detection was developed.

EXPERIMENTAL

Reagents and Materials—The Compound I and *dl*-2-(2-methoxy-4-biphenylyl)propionic acid (II) used in this study were synthesized[1]. Methyl ethyl ketone (III), acetone, and cyclohexane, distilled in glass, were used as supplied[2]. Stock solutions of I in III (100 μg/ml), II in III (100 μg/ml), pentafluorobenzyl bromide[3] in acetone (25 μg/ml), and aqueous sulfuric acid (1 *N*) were stored in glass containers. All other solvents were analytical reagent grade. Silicone gum rubber (OV-17) on 80–100-mesh Gas Chrom Q (3% w/w) was used as supplied[4].



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Instrumentation—A two-speed reciprocating shaker[5] was used for shaking the samples in the horizontal position. A mixer[6] was used to aid in eluting the zones removed from thin-layer chromatograms. GLC measurements were made with a gas chromatograph[7] equipped with a hydrogen flame-ionization detector, a nickel-63 electron-capture detector, and a −0.2 to 1.0-mv recorder[8]. All cylinders of gases used for chromatography (*i.e.*, helium, hydrogen, oxygen, and 5% methane in argon) were fitted with filters containing molecular sieve 4A.

TLC—All analytical chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[9], ascendingly developed in a solvent system composed of 20% (v/v) acetic acid in toluene. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp. Under these conditions, I and II had the same R_f values, *i.e.*, 0.45. For preparative and analytical chromatography, during the isolation of the II-pentafluorobenzyl ester, a solvent system composed of hexane–acetone–methanol (100:10:10) was utilized. The R_f values for I- and II-pentafluorobenzyl esters were identical, *i.e.*, 0.42.

GLC Conditions—All chromatography was conducted using U-shaped glass columns [0.61 m (2 ft) × 3 mm i.d.] packed with 3% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained isothermally at 210, 250, and 270°, respectively. Flow rates of 5% methane in argon, used as carrier and purge gas, were maintained at 50 ml/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, I- and II-pentafluorobenzyl esters had retention times of 4.2 and 7.4 min, respectively (Fig. 1). In ancillary studies, using the flame-ionization detector, flow rates for helium, hydrogen, and oxygen were maintained at 50, 40, and 300 ml/min, respectively.

Synthesis of Standard Materials—Place 200 mg I in a 15-ml glass-stoppered centrifuge tube. Add 8 ml acetone, 0.4 ml undiluted pentafluorobenzyl bromide, and 113 mg anhydrous potassium carbonate. Stopper the centrifuge tube and heat in a water bath at 60° for 16 hr. Evaporate to dryness with a gentle stream of nitrogen gas. Add 2 ml water and 5 ml cyclohexane. Shake for 10 min and allow the phases to separate. Transfer the cyclohexane to a fresh 15-ml centrifuge tube. Repeat the extraction with two additional 5-ml portions of cyclohexane, and evaporate the pooled cyclohexane extracts to dryness with nitrogen gas.

Reconstitute the cyclohexane extract residue in 10 ml of a solvent mixture composed of toluene–acetic acid (80:20) (v/v). Apply the solution to a silica gel column (2.8 × 50 cm) and develop at a flow rate of 3 ml/min with a solvent system composed of toluene–acetic acid (80:20) (v/v). Collect 10-ml fractions and monitor each fraction by TLC *(vide supra)*. Pool fractions 10–25 and remove the solvent under reduced pressure (15 mm).

[1] Research Division, Boots Pure Drug Co. Ltd., Nottingham, England.

[2] Burdick and Jackson Labs., Muskegon, Mich.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Applied Science Labs., State College, Pa.
[5] Eberbach and Sons, Ann Arbor, Mich.
[6] Vortex model K-500, Scientific Industries, Queen's Village, N.Y.
[7] F&M model 400, Hewlett-Packard Co., Avondale, Pa.
[8] Honeywell Elektronic 15.
[9] Brinkmann Instruments, Westbury, N.Y.

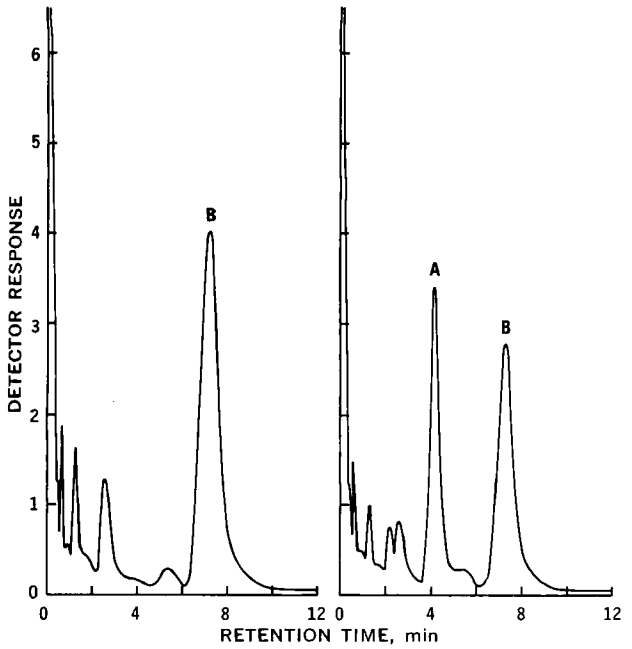


Figure 1—*Gas–liquid chromatograms (electron-capture detector) of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 1 hr after single-dose oral administration of 10 mg flurbiprofen. Key: A, flurbiprofen pentafluorobenzyl ester; and B, II-pentafluorobenzyl ester.*

Synthesize standard material for identification of II-pentafluorobenzyl ester, utilizing the same general reaction conditions as described for I. Reconstitute the cyclohexane extract residue in 10 ml of the column chromatography solvent system (*vide infra*). Apply the solution to a silica gel column (2.8 × 50 cm) and develop at a flow rate of 3 ml/min with a solvent system composed of hexane–acetone–methanol (100:10:10). Collect 10-ml fractions and monitor each fraction by TLC (silica gel F_{254}) in hexane–acetone–methanol (100:10:10). Pool fractions 20–32 and remove the solvent under reduced pressure (15 mm). Recrystallize from methanol–ether.

Assay Procedure—*Preparation of Plasma Standards*—Pipet 1-ml aliquots of the I–III stock solution, diluted to 0.1, 0.5, 1, 3, and 5 μg/ml, into glass-stoppered centrifuge tubes. Add a 1-ml aliquot of the II–III stock solution, diluted to 4 μg/ml, to each centrifuge tube. Evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma specimens.

Preparation of Samples—Pipet 1-ml aliquots of the II–III stock solution, diluted to 4 μg/ml, into a series of glass-stoppered centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen gas, and place 1 ml of plasma into each centrifuge tube. Add 0.25 ml 1 *N* aqueous sulfuric acid and 5 ml benzene, and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 4-ml aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml chloroform and evaporate to dryness with nitrogen.

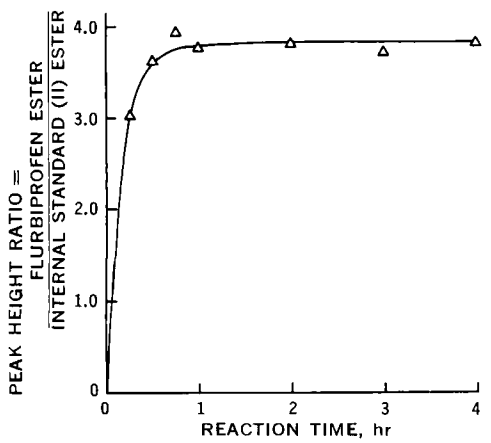


Figure 2—*Effect of reaction time on formation of flurbiprofen pentafluorobenzyl ester.*

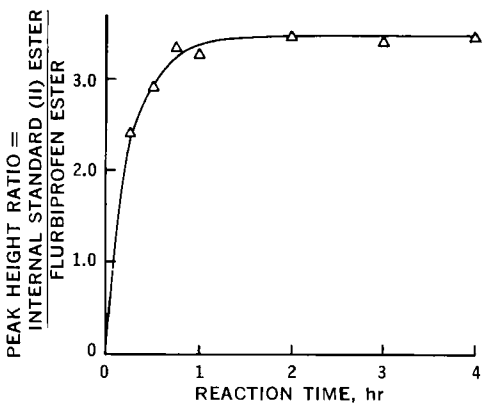


Figure 3—*Effect of reaction time on formation of internal standard pentafluorobenzyl ester (II-pentafluorobenzyl ester).*

Reconstitute the benzene extract residues from the plasma standards and samples in 50 μl chloroform. Together with appropriate standards, spot all of each sample on thin layers of silica gel F_{254} (*vide supra*). Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms thoroughly at room temperature (24°), and visualize the zones by irradiation with a 254-nm UV lamp. Scrape the zones corresponding to I into separate glass-stoppered centrifuge tubes. Add 2 ml methanol to each tube and gently agitate using the mixer. Centrifuge for 5 min at 2000 rpm. Transfer 1 ml of the methanol layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen gas.

Add 25 mg anhydrous potassium carbonate and a 2-ml aliquot of the pentafluorobenzyl bromide stock solution to each centrifuge tube. Stopper tightly and heat in a water bath at 60° for 90 min. Evaporate to dryness with nitrogen gas. Add 1 ml water and 1 ml cyclohexane, shake manually for 1 min, and allow the phases to separate. Inject a 2-μl aliquot of the cyclohexane layer for analysis into the chromatograph (operating in the electron-capture detector mode).

Calculations—The peak heights for I- and II-pentafluorobenzyl esters are measured. Peak height ratios are obtained by dividing the peak height of the I-pentafluorobenzyl ester by the peak height of the II-pentafluorobenzyl ester. Calibration curves from known concentrations of I in plasma are prepared by plotting peak height ratios *versus* free acid concentration, expressed as micrograms per milliliter plasma. Values for unknown concentrations of I in plasma specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—Informed written consent was obtained from each of six normal human male volunteers prior to participation in this study. All subjects were between the ages of 21 and 55 years and ranged in body weight from 62.3 to 83.2 kg and in height from 1.73 to 1.85 m. All subjects fasted for 16 hr prior to drug administration. Each subject received a 10-mg dose of I as a compressed tablet. Food was withheld for an additional 2 hr. Blood specimens (10 ml) were withdrawn into heparinized syringes at predetermined time intervals from 0 to 24 hr after drug administration. The plasma was harvested and stored at −18°.

RESULTS AND DISCUSSION

Synthesis and Identification of Pentafluorobenzyl Esters—Investigations by Kawahara (5, 6) indicated that pentafluorobenzyl bromide was a useful reagent for the microdetermination of

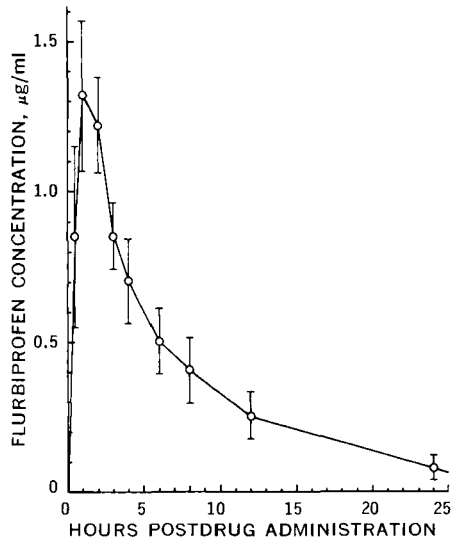


Figure 4—*Mean* (±SEM) *plasma concentrations of flurbiprofen* versus *time in humans* (n = 6) *after single-dose oral administration of 10 mg drug as a compressed tablet.*

phenols, mercaptans, and organic acids in water. The general reaction for the preparation of pentafluorobenzyl esters from carboxylic acids is presented in Scheme I. A series of samples, containing known amounts of I and II, were prepared to determine optimal reaction times for formation of the pentafluorobenzyl esters. In studies with I, known amounts of II-pentafluorobenzyl ester were added as internal standard. Similarly, in studies with II, known amounts of I-pentafluorobenzyl ester were added as internal standard. The results indicated that ester formation for both compounds was completed within 90 min (Figs. 2 and 3). In the presence of water and cyclohexane, the derivatives were stable for at least 48 hr.

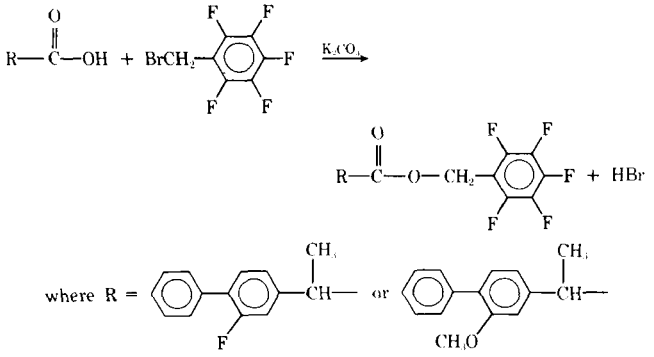
Synthesis of standard material indicated that the pentafluorobenzyl ester of I was a liquid at room temperature. Attempts to isolate crystalline material were unsuccessful. GLC, using a solid sample injector and flame-ionization detector, indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{22}H_{14}F_6O_2$: C, 62.27; H, 3.33; F, 26.87. Found: C, 62.26; H, 3.86; F, 26.70.

The pentafluorobenzyl ester of II was isolated as fine white crystals, mp 53–55°. GLC, using a solid sample injector and flame-ionization detector, indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{23}H_{17}F_5O_3$: C, 63.30; H, 3.93; F, 21.77. Found: C, 63.43; H, 3.91; F, 21.31.

IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the pentafluorobenzyl esters of I and II chromatographed as the intact molecules.



Scheme I—Preparation of pentafluorobenzyl esters from carboxylic acids

Table I—Recovery of Flurbiprofen from Human Plasma[a]

Added, μg/ml	Found, μg/ml	Recovery, %
0.10	0.100	100.0
0.50	0.563	112.6
1.00	0.860	86.0
3.00	2.871	95.7
5.00	4.510	90.2
10.00	9.792	97.9
	Mean ± *SD*	97.1 ± 9.2

[a] *dl*-2-(2-Methoxy-4-biphenylyl)propionic acid utilized as internal standard.

TLC of Plasma Extracts—Pilot studies were conducted with various TLC solvent systems to separate materials extracted from plasma which interfered in the direct GLC measurement of II. A solvent system composed of 20% (v/v) acetic acid in toluene separated two materials from human plasma extracts, designated zone A (R_f 0.16) and zone B (R_f 0.31), from known I and II (R_f values 0.45). The materials were eluted from the silica gel and reacted with pentafluorobenzyl bromide. Based upon GLC behavior of the esters, on a column of 3% (w/w) OV-17, the material in zone A (R_f 0.16) was responsible for the interference observed in the analysis of II. No interference from zone B was observed in the GLC analysis of I or II.

Assay Sensitivity and Specificity—At a sensitivity of 3.2×10^{-10} amp/mv, 0.0028 μg of I as its pentafluorobenzyl ester produced a full-scale response. However, under the assay conditions, the lower limit of detection sensitivity of I in extracts of human plasma is 0.05 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of 0–10 μg/ml. Quantification from a standard curve was adequate. Analysis of plasma specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay was identical to known I-pentafluorobenzyl ester.

Recovery Experiments—Known amounts of I and II, in III, were evaporated to dryness in centrifuge tubes, and water or plasma was added. The samples were thoroughly mixed and extracted with benzene. All extract residues were chromatographed on thin layers of silica gel F_{254}, and the zones corresponding to I and II were eluted. The materials were esterified and analyzed *via* GLC. The results (Tables I and II) indicated that recoveries of I (97.1 ± 9.2%) and II (98.3 ± 3.9%) from plasma were essentially quantitative as compared to simple aqueous samples.

Plasma Levels of Flurbiprofen in Humans—Results from the measurement of plasma I concentrations in six normal human subjects, after single-dose oral drug administration, demonstrated the utility of the analytical methodology (Fig. 4). A peak mean *(±SEM)* level of I (1.32 ± 0.25 μg/ml) was observed at 1 hr after drug administration, indicating rapid drug absorption from the compressed tablet formulation. Only trace amounts of intact drug (0.07 ± 0.04 μg/ml) were found in the 24-hr plasma specimens, suggesting rapid drug disappearance from peripheral circulation. A plot of the logarithms of the average plasma I concentrations,

Table II—Recovery of Internal Standard (II)[a] from Human Plasma[b]

Added, μg/ml	Found, μg/ml	Recovery, %
0.10	0.094	94.0
0.50	0.479	95.8
1.00	1.015	101.5
3.00	2.838	94.6
5.00	5.095	101.9
10.00	10.200	102.0
	Mean ± *SD*	98.3 ± 3.9

[a] Internal standard (II) = *dl*-2-(2-methoxy-4-biphenylyl)propionic acid. [b] Flurbiprofen utilized as internal standard.

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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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).