Binding of Disopyramide to α_1-Acid Glycoprotein in Plasma Measured by Competitive Equilibrium Dialysis

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Abstract □ A modification of equilibrium dialysis in which α_1-acid glycoprotein and plasma compete directly for disopyramide has been used in conjunction with binding curves to measure the extent of the α_1-acid glycoprotein–disopyramide interaction. At concentrations in the therapeutic range, 80–90% of disopyramide was bound to α_1-acid glycoprotein for plasma from each of six healthy adults. Also, equilibrium dialysis data are presented, indicating that pH does not influence the binding of disopyramide within the therapeutic range.

Keyphrases □ Disopyramide—binding to α_1-acid glycoprotein, competitive equilibrium dialysis □ Equilibrium dialysis, competitive—binding of disopyramide to α_1-acid glycoprotein □ α_1-Acid glycoprotein—binding of disopyramide, competitive equilibrium dialysis

The antiarrhythmic drug disopyramide has concentration-dependent plasma protein binding (1) and variability in therapeutic response which may be due, at least in part, to variability in protein binding. An increased number of reports have associated the binding of basic drugs with α_1-acid glycoprotein (2–10). Interindividual variability in the free concentration of the drug and its effect could conceivably be produced by differing concentrations of this protein, as even the reference range for α_1-acid glycoprotein is wide (55–140 mg/dL). As α_1-acid glycoprotein is an acute phase reactant that can approximately double in concentration following acute myocardial infarction (11, 12), its involvement in disopyramide binding may be of even greater significance in this situation.

In previous studies, parameters obtained either from Scatchard plots or the bound fraction of the drug in buffered solution of protein alone were compared with data obtained in plasma (5, 8–10). This approach was also used to implicate α_1-acid glycoprotein in the binding of disopyramide by David *et al*[1]. Others have implied the relationship from correlations of plasma protein concentration with the extent of protein binding (2–4, 6).

To assess the contribution of a specific protein to the total binding occurring in plasma, a more direct evaluation of the interaction would be desirable. The work presented here utilizes a procedure in which the moles of drug bound to protein and to macromolecules in plasma are determined while both protein and plasma compete for the drug. As this procedure is essentially a variation of equilibrium dialysis, we have called the method competitive equilibrium dialysis. The effect of pH on the binding has also been investigated because of its potential importance in binding results.

EXPERIMENTAL

Reagents—The buffer used was 0.1 M Na_2HPO_4 (0.3% NaCl) unless otherwise stated; it was prepared from analytical-grade reagents and distilled water[2]. Disopyramide and the ^{14}C-radiolabeled disopyramide were supplied[3]. The [^{14}C]disopyramide was further purified by HPLC and stored at 4°C in 0.1 M pH 7.4 Na_2HPO_4 buffer containing 0.3% NaCl. The radiochemical purity of the purified disopyramide was >98%. Human α_1-acid glycoprotein purified from Cohn's fraction VI was obtained commercially[4] and used without further treatment. A stock solution of α_1-acid glycoprotein was prepared in buffer, and the concentration was determined from the molar absorbance at 278 nm. Appropriate dilutions were then used to provide all α_1-acid glycoprotein solutions including standards for assays by radial immunodiffusion. Comparison of these standards with commercially available standard human serum[5] indicates the glycoprotein to be >90% pure.

Scintillation Counting—Sufficient [^{14}C]disopyramide was used in all samples to ensure more than 1000 cpm/vial. For all samples, 200-μL duplicates were placed in respective polyethylene vials followed by 2 mL of tissue solubilizer[6]. After mixing and standing for 30 min, 12 mL of scintilation fluid[7] containing 100 μL of glacial acetic acid was added before capping and further mixing. Scintillation counting was carried out until at least 10,000 counts were accumulated using a scintillation counter[8] with external standardization. Means of duplicate counts were used for all calculations. Samples were counted before dialysis to determine specific activities as well as sampling contents of each dialysis compartment at equilibrium.

α_1-Acid Glycoprotein Determinations—Quantitation of α_1-acid glycoprotein employed the method of Mancini *et al.* (13) using radial immunodiffusion plates[9]. Four standards were run per plate. Interassay variability was evaluated by repeated analysis of a single sample, and accuracy was estimated by duplicate analysis of standard human serum containing 92 mg/dL of the glycoprotein[8].

Dialysis—Blood from six healthy volunteers was obtained by venipuncture in plain glass syringes and immediately transferred to EDTA tubes from which the stoppers had been discarded. Plasma was obtained ~30 min later after centrifugation, and the samples assayed for α_1-acid glycoprotein before being stored at −18°C in glass tubes with polytef-lined screw caps.

Each of 20 polymethyl methacrylate dialysis cells having two 1-mL compartments were prepared using dialysis membrane[10] that had been soaked in distilled water for 30–60 min. The experiment consisted of three sections: First, plasma from each of the subjects was dialyzed against six different concentrations of disopyramide in pH 6.7 buffer (pH 7.2 at equilibrium). These results were used to prepare a binding curve for each subject. The second section involved competitive equilibrium dialysis. This was experimentally identical to the first section except sufficient glycoprotein was added to the buffer to produce approximately equal concentrations in opposing compartments of each cell at equilibrium. In the third section, the effect of pH on the binding was evaluated. Three of the disopyramide concentrations used in the binding curve were prepared in pH 6.4 buffer (equilibrium pH 6.9) and pH 7.2 buffer (equilibrium pH 7.6). These solutions were then also dialyzed against plasma. By also utilizing the data from the binding curve, three disopyramide concentrations at three pH values were investigated.

Dialysis was carried out for 5 h at 37°C. These dialysis conditions have been shown to produce equilibrium for disopyramide concentrations (at equilibrium in the plasma) across the range of 1.0–18 mg/L for equilibrium dialysis and across the range of 2.5–10 mg/L during competitive equilibrium dialysis. On conclusion of dialysis in all three sections of the experiment, the contents of each compartment were transferred to glass vials using a 500-μL syringe[11].

[1] B. M. David, E. G. Whitford and K. F. Ilett, Presentation at the 15th annual meeting of the Australasian Society of Clinical and Experimental Pharmacologists, Adelaide, Australia, December 1981.

[2] Milli-Q System; Millipore Pty. Ltd., North Ryde, NSW, Australia.
[3] Hoechst-Roussel Pharmaceuticals Pty. Ltd., Sydney, Australia.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Behring Institute, Marburg, W. Germany.
[6] NCS; Amersham Aust. Pty. Ltd., Surrey Hills, NSW, Australia.
[7] PCS II; Amersham Aust. Pty. Ltd., Surrey Hills, NSW, Australia.
[8] Model 2660; Packard Instrument Co., Downers Grove, Ill.
[9] Calbiochem Behring Aust. Pty. Ltd., Carlingford, NSW, Australia.
[10] Spectrapor II; Spectrum Medical Industries, Los Angeles, Calif.
[11] Hamilton Co., Reno, Nev.

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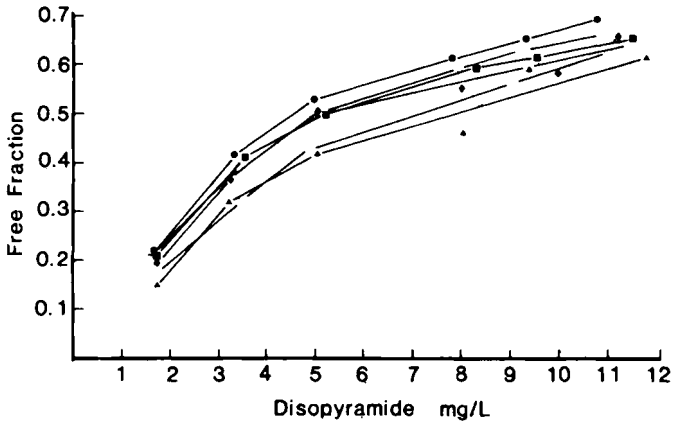

Figure 1—*Relationship of free fraction of disopyramide in plasma to total disopyramide concentration curves for disopyramide. No mathematical curve fitting procedures were used. Key: (■) subject 1; (▲) subject 2; (●) subject 3; (◆) subject 4; (□) subject 5; (○) subject 6.*

The contents of each compartment were then sampled for scintillation counting and α_1-acid glycoprotein assay.

To provide sufficient volume to determine pH, samples were combined in the following manner: For samples from sections 1 and 2, the contents of the plasma side of the dialysis unit from the three lowest disopyramide concentrations were combined; the four highest concentrations were also combined. A similar combination regimen was used for the samples from the buffer side. For samples from section 3, all three concentrations were combined, again keeping plasma side and buffer side samples separate.

RESULTS AND DISCUSSION

Interassay coefficient of variation for α_1-acid glycoprotein assays was 2.2% (n = 17; mean = 84 mg/dL). The results of duplicate analyses of standard human serum (92 mg/dL) were 97 and 100 mg/dL, indicating the accuracy of this method. Ratios of volumes of buffer compartment to plasma compartment at equilibrium were determined for all cells. The mean values ($\pm SD$) of this ratio for all cells dialyzed was 0.92 ± 0.05, illustrating the osmotic effect of the macromolecular components of plasma, as isotonic solutions were used. Total disopyramide concentrations at equilibrium and free fractions were calculated from equilibrium radiolabel concentrations and initial specific activities as per Norris *et al.* (14).

The curves of free fraction of disopyramide against total disopyramide concentration are shown as Fig. 1. These were then used as standard curves to determine the free fraction of disopyramide present after competitive dialysis in respective plasma samples at their equilibrium concentrations. From the free fraction and total drug concentration, the free and bound concentrations in the plasma were calculated. As the free concentration is the same in both compartments at equilibrium, the bound concentrations in the buffer side were also readily calculated. The contribution of α_1-acid glycoprotein to the binding in the plasma was calculated by Eq. 1. This equation assumes equal glycoprotein concentrations in both compartments at equilibrium and, under such conditions, provides direct quantitation of the relative importance of α_1-acid glycoprotein in the plasma protein binding of disopyramide:

$$\text{Fraction of binding attributable to } \alpha_1\text{-acid glycoprotein} = \frac{DISO_\beta^b}{DISO_\beta^p} \qquad \text{(Eq. 1)}$$

where $DISO_\beta^b$ and $DISO_\beta^p$ are the concentrations of disopyramide bound at equilibrium in the buffer and plasma compartments, respectively.

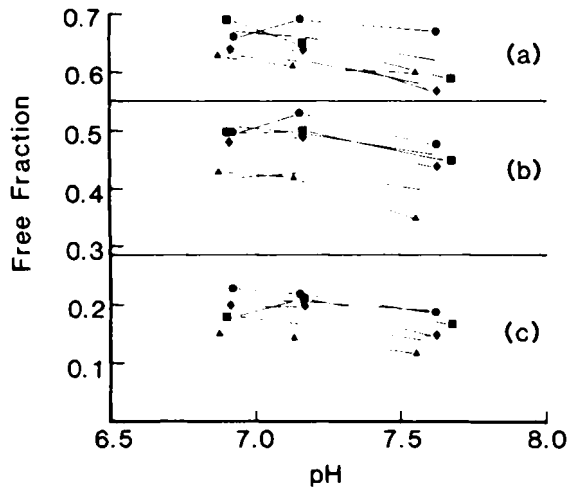

Figure 2—*Effect of pH on the binding of disopyramide to plasma. Symbols as for Fig. 1. Mean (and range) of disopyramide concentrations in plasma equilibrium: (a) 11.2 (10.5–11.8) mg/L; (b) 5.06 (4.74–5.27) mg/L; (c) 1.63 (1.3–1.75) mg/L.*

Table I—Binding Curves and Competitive Equilibrium Dialyses of α_1-Acid Glycoprotein at Equilibrium[a]

Subject	Binding Curve, Plasma	Competitive Equilibrium Dialysis Buffer	Competitive Equilibrium Dialysis Plasma
1	79.8 ± 2.2	77.3 ± 4.3	77.1 ± 3.2
2	80.6 ± 2.5	85.1 ± 2.7	75.9 ± 3.2
3	57.0 ± 1.1	64.4 ± 1.9	59.5 ± 1.2
4	67.6 ± 0.6	75.1 ± 1.0	67.0 ± 2.3
5	84.6 ± 1.6	85.9 ± 2.3	84.4 ± 1.5
6	72.3 ± 2.1	75.7 ± 3.1	73.0 ± 2.5

[a] Mean ± SD (mg/dL); n = 6 for binding curve studies and n = 5 for the competitive equilibrium dialyses.

Table II—Ratios of Disopyramide Bound to α_1-Acid Glycoprotein/ Disopyramide Bound to Plasma, Determined by Competitive Equilibrium Dialysis

Subject	Plasma Disopyramide at Equilibrium, mg/L 2.6	4.2	6.7	8.1	9.9
1	0.85	—[a]	—[a]	0.78	0.86
2	0.85	0.75	0.83	0.74	0.80
3	0.77	0.71	0.80	0.67	0.73
4	0.82	0.78	0.84	0.77	0.83
5	0.96	0.88	0.93	0.86	0.81
6	1.05	0.90	0.88	0.84	0.88

[a] —Missing value.

Table I reports the mean values of α_1-acid glycoprotein concentrations of compartments at equilibrium for each of the subjects. As these results do not show exact agreement in the glycoprotein concentration between compartments, some comment regarding subsequent errors in data obtained from Eq. 1 is necessary. With the relatively narrow range of α_1-acid glycoprotein concentrations being considered for plasma from each subject, it is reasonable to assume that the moles of drug bound varies linearly with the protein concentration. The percent error in data obtained using Eq. 1 is therefore given by:

$$\%\ \text{error} = 1 - \frac{[\alpha_1 AG]^b}{[\alpha_1 AG]^p} \times 100 \qquad \text{(Eq. 2)}$$

where $[\alpha_1 AG]^b$ and $[\alpha_1 AG]^p$ are the concentrations of α_1-acid glycoprotein in the buffer and plasma compartments at equilibrium, respectively.

Application of Eq. 2 to the data in Table I indicates the errors associated with the calculations from Eq. 1 to be 0.3, 12, 8, 12, 2, and 4%, respectively, for subjects 1–6. Data obtained using Eq. 1 is presented as Table II, showing that α_1-acid glycoprotein is responsible for ~80–90% of the binding of disopyramide at concentrations from 2.6 to 9.9 mg/L. Therefore, this glycoprotein is the major binding protein in plasma for disopyramide at therapeutic concentrations.

The free fraction of drug at each of three pH values for three different concentrations is presented as Fig. 2. Variations in pH within the physiological range have no important effect on the protein binding of disopyramide.

REFERENCES

(1) P. J. Meffin, E. W. Robert, R. A. Winkel, S. Harapat, F. A. Peters, and D. G. Harrison, *J. Pharmacokinet. Biopharm.*, **7**, 29 (1979).

(2) K. M. Piafsky, O. Borga, I. Odar-Cederlof, C. Johnansson, and J. Sjoqvist, *N. Engl. J. Med.*, **299**, 1435 (1978).

(3) R. L. Nation, *Clin. Pharmacol. Ther.*, **29**(4), 472 (1981).

(4) M. Wood and A. J. J. Wood, *Clin. Pharmacol. Ther.*, **29**(4), 522 (1981).

(5) M. Brinkschulte and V. Breyer Pfaff, *Arch Pharmacol.*, **314**, 61 (1980).

(6) M. K. Romach, K. M. Piafsky, J. G. Abel, V. Khouw, and E. M. Sellars, *Clin. Pharmacol. Ther.*, **29**(2), 211 (1981).

(7) K. M. Piafsky, *Clin. Pharmacokinet.*, **5**, 246 (1980).
(8) E. Pike, B. Skuterud, P. Kierulf, D. Fremstad, S. M. Abdel Sayed, and P. K. M. Lunde, *Clin. Pharmacokinet.*, **6**, 367 (1981).
(9) P. J. McNamara, R. L. Slaughter, J. A. Pieper, M. G. Wyman, and D. Lalka, *An. Analg.*, **60**, 295 (1981).
(10) D. Fremstad, K. Bergerud, J. F. W. Haffner, and P. K. M. Lunde, *Eur. J. Clin. Pharmacol.*, **10**, 411 (1976).
(11) B. G. Johansson, C. O. Kindmark, E. Y. Trell, and F. A. Wollheim, *Scand. J. Clin. Lab. Invest.*, **291** (suppl) 124, 117 (1972).
(12) S. J. Smith, G. Bos, M. R. Esseveld, H. G. Van Eijk, and J. Gerbrandy, *Clin. Chim. Acta.*, **81**, 75 (1977).
(13) G. Mancini, A. O. Carbonara, and J. F. Hereman, *Immunochemistry*, **2**, 235 (1965).
(14) R. L. G. Norris, J. T. Ahokas, and P. J. Ravenscroft, *J. Pharmacol. Meth.* **7**, 143 (1982).

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In Vitro and *In Vivo* Assessment of Hepatic and Extrahepatic Metabolism of Diazepam in the Rat

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Abstract □ Since diazepam is metabolized by many organs in the rat, the microsomal fractions of the liver, kidney, and lung from male Wistar rats were assayed for NADPH-dependent metabolism of diazepam and enzymatic parameters. The predicted extraction ratios were obtained from this *in vitro* experimental system. The organ clearances of the liver, kidney, and lung were then calculated for the determination of the relative contribution of each eliminating organ to the total body clearance (CL_{tot}) of diazepam in the rat. The liver was the most effective eliminating organ, followed by the kidney and the lung, in that order. The hepatic extraction ratio of diazepam was determined *in vivo* after portal and femoral vein administrations of diazepam. The validity of the *in vitro* experimental system for the liver was demonstrated by a good agreement between the calculated hepatic extraction ratio of diazepam from *in vitro* enzymatic parameters (0.616) and that derived *in vivo* (0.648). However, the sum of organ clearances of the liver, kidney, and lung did not account for CL_{tot} of diazepam in the rat, suggesting the possible contribution of the metabolism in the other organs or tissues, or an underestimation of the pulmonary and renal metabolism.

Keyphrases □ Diazepam -metabolism, hepatic and extrahepatic, *in vitro* assessment, comparison with *in vivo* parameters in the rat □ Metabolism—diazepam in the rat, hepatic and extrahepatic, *in vitro* assessment compared with *in vivo* parameters

Diazepam is a clinically important minor tranquilizer which is extensively metabolized by all species studied. However, there are pronounced interspecies differences in the total plasma clearance (CL_{tot}) per unit of body weight that presumably reflect differences in organ intrinsic clearances, blood flow, plasma binding, and the relative contributions of eliminating organs (1). In humans the total clearance is small [26.6 ± 4.1 mL/min (mean ± *SD*; $n = 5$)] (2) and it is assumed to reflect only hepatic metabolism. In contrast, in the rat the total clearance exceeds the liver blood flow, suggesting the involvement of extrahepatic elimination. The relative contribution of different organs to the overall metabolism of a drug may be assessed *in vivo*, but this is often difficult. Instead, comparison is often made between the *in vitro* enzymatic characteristics of an organ. This approach has recently received new impetus, since it has been shown to be readily extrapolated to the whole organ.

In the present study, the *in vitro* experimental system using microsomal fractions was employed to evaluate enzymatic parameters of the hepatic and extrahepatic diazepam elimination systems in the rat. The kidney and the lung were selected as the representative extrahepatic eliminating organs, since these two organs are reported to contain considerable amounts of cytochrome P_{450} (3). Enzymatic parameters of the liver, kidney, and lung were extrapolated to those of the whole organs. Using these parameters, the relative contributions of individual disposing organs were evaluated and, in the case of the liver, compared with an *in vivo* assessment.

EXPERIMENTAL

Materials—Male Wistar rats[1], weighing 250–270 g, on a normal laboratory diet were used throughout. [2-^{14}C]Diazepam (40–60 mCi/mmoL)[2] was used. Unlabeled diazepam[3], demethyldiazepam[4], oxazepam[4], and 3-hydroxydiazepam[4] were gifts from commerical sources. NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained commercially[5]. All other chemicals were reagent grade.

Assay of Radioactivity—[2-^{14}C]Diazepam was diluted with unlabeled drug prior to the studies. The determination of carbon-14 and the assay of labeled drug and its metabolites were described elsewhere (4). The radiochemical purity was >98–99% for [2-^{14}C]diazepam by TLC.

Tissue Preparation and Drug Metabolism Study—Each of the 3–5 separate determinations were conducted on a separate pool of organs, each pool containing organs from 2–10 individual animals. After overnight fasting, the rats were exsanguinated *via* a carotid artery and perfused *in situ* with cold physiological saline *via* the venous trunk just inferior to the renal veins and *via* the portal vein until the effusate became colorless. The liver, kidney, and lung were excised, blotted, weighed, and pooled to obtain 10–11 g of each organ. The preparation of microsomal fractions was described elsewhere (5).

Incubation mixtures for the mixed function oxidation consisted of microsomal protein, 50 mM Tris-HCl buffer (pH 7.4), the NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride), and various amounts of [^{14}C]diazepam diluted with unlabeled diazepam to a final volume of 1.0 mL. The NADPH-generating system, microsomal protein solution, and [^{14}C]diazepam solution were preincubated separately for 2 min at 37°C. The

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[2] Radiochemical Center, Amersham, England.
[3] Yamanouchi Pharm. Co., Tokyo, Japan.
[4] Banyu Pharm. Co., Tokyo, Japan.
[5] Boehringer Mannheim Yamanouchi Co., Tokyo, Japan.

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