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LIPOPROTEIN AND PROTEIN BINDING OF THE CALCIUM CHANNEL BLOCKER DILTIAZEM

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ABSTRACT. The plasma protein binding properties of the calcium channel blocker diltiazem were studied using a three-chamber equilibrium dialysis system. Diltiazem is 81% bound to human sera with significant inter-individual variation. The relative binding of diltiazem by lipoproteins and α_1-acid glycoprotein was higher than by albumin. The binding to low density lipoprotein was strong and appeared not to be associated with the surface apoprotein.

Many drugs circulate highly bound to plasma proteins (1). It is generally assumed that the unbound or the free drug fraction is the pharmacologically active fraction, and in several instances plasma free drug concentrations have been shown to reflect drug effects better than plasma total drug concentrations (2). Thus, for highly protein-bound drugs, altered protein binding can affect the effectiveness of drug therapy.

The calcium channel blockers (CABs) verapamil, nifedipine, and diltiazem are highly protein bound (>80%) (3,4). There is evidence that α-1-acid glycoprotein (AAG) and lipoproteins (LPs) bind verapamil and nifedipine, respectively(5,6). The plasma proteins which bind diltiazem, however, have not been identified (4). We are interested in the protein binding properties of the CABs because these drugs have assumed a major therapeutic role in the management of patients with cardiovascular diseases. Furthermore, this population of patients have an increased incidence of hyperlipoproteinemias. If CAB binding to lipoproteins is extensive, then the free drug fraction will be highly variable since the degree of hyperlipoproteinemia can change rapidly with diet and clinical status of the patient.

We report here the plasma protein binding properties of diltiazem hydrochloride, (D-3-acetyoxy-cis-2,3-dihydro-5-(2-dimethylamino-ethyl)-2(p-methoxyphenyl)-1,5-benzothiazepin-4-(5H)-one hydrochloride). Our data show that the relative binding of diltiazem to LP fractions and AAG were higher than that to albumin.

MATERIALS AND METHODS. α-1-acid glycoprotein (98% pure) was purchased from Calbiochem-Behring. Human serum albumin (Cohn fraction V) was prepared by the Rochester Red Cross. Diltiazem and ^{14}C-diltiazem were gifts from Marion Laboratories. ^{14}C-diltiazem radiopurity(>98%) was verified by thin layer chromatography with the solvent system: chloroform-ethanol-n-butanol-water-formic acid: acetic acid (100:50:10:5:5:3). The polyclonal and monoclonal immunoglobulins were gifts from Dr. George Abraham, Strong Memorial Hospital. Lipoprotein (LP) fractions including very low, low and high density LPs (VLDL, LDL, and HDL, respectively) were isolated from human serum by the sequential density ultracentrifugation procedure of Hatch and Lees (7). LP total protein determination in the presence of 4% sodium dodecyl sulfate was a modified Lowry procedure (8).

Protein binding was studied by equilibrium dialysis at 37°C in Sorenson's isotonic phosphate buffer using three-chamber dialysis cells (Technilab). Proteins in outside chambers A and C were separated from diltiazem in middle chamber B (160-16,000 ng/ml, 15,000 cpm) by two Spectra-Por 2 dialysis membranes (Spectrum). At specified times, radioactivity in each of the chambers was

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determined by liquid scintillation counting. Quenching was corrected by the channel-ratio method. The bound fraction is calculated by the relationship:

$$\frac{\text{total drug (A or C)} - \text{Free drug (B)}}{\text{total drug (A or C)}}$$

Diltiazem binding to LDL was studied by incubating duplicate tubes of LDL (1.5 mg/ml) with ^{14}C-diltiazem (800 ng/ml, 30,000 cpm) in 1 ml for various times up to 25 hours at 37°C. At the end of each incubation period, 0.3 ml of 2% pronase and 5 ul of $CaCl_2$(1M)were added to one tube whereas only buffer and $CaCl_2$ were added to its control tube. After incubation (37°C) for one hour, 0.4 ml of each tube was withdrawn to mix with 0.1 ml of 5% human serum albumin and 0.5 ml of cold 20% trichloroacetic acid (TCA). After standing for one hour, TCA precipitable material was pelleted by centrifugation and the supernatants were decanted and pooled with TCA washings of the pellets for radioassay. The TCA pellets were extracted with 2.5 ml of cold chloroform/methanol (2:1, V/V) and 0.5 ml of buffer/0.15M NaCl. After centrifugation, the lower organic phase was removed for radioassay.

To the remaining incubation solution, cold NaBr (density=1.126 g/ml)was added to bring the density to 1.063g/ml. The tubes were chilled in ice before application of an overlay of 2.0 ml of NaBr (d=1.063 g/ml) at room temperature. LDL was then floated by ultracentrifugation at 4°C, 50,000 rpm for 18 hours using a 75Ti rotor in a Beckman L8-70 model centrifuge. The top 2 ml of the supernatants ("LDL" fractions) were removed and radioassayed.

Venous blood from patients and healthy volunteers was collected into "Vacutainer tubes" which do not contain the plasticizer tris-(2-butoxyethyl)-phosphate. Serum cholesterol and triglyceride levels were analyzed by the Clinical Chemistry Laboratory.

RESULTS

Validation of equilibrium dialysis system. The time required to reach equilibrium was determined using a 1:2 dilution of plasma (33 mg/ml) in chamber A, ^{14}C-diltiazem (100 ng, 17 DPM/ng) in buffer in B and buffer alone in C. The radioactivity in each chamber was determined at specified times. As expected, at equilibrium which occurred after six hours, the amount of drug was the same in Chambers B and C. It was significantly lower than that in Chamber A containing plasma, indicating substantial diltiazem binding to plasma proteins. The fraction of diltiazem bound to the 1:2 diluted plasma is 0.48 $\pm$ 0.02 (SD).

Non-specific adsorption of diltiazem to the dialysis membranes in 9 experiments was 3.1% $\pm$ 0.32 (SE) and was independent of diltiazem concentration and the protein contents of chambers A and C.

The Donnan effect at high protein concentrations was assessed by measuring the binding of diltiazem to albumin(27.4 mg/ml) with constant free diltiazem concentration (3535 $\pm$ 53 ng/ml) and increasing concentrations of NaCl (0 to 0.5M at 0.1M increments). Increasing NaCl concentrations did not change the bound fraction (25.4 $\pm$ 1.27) (SD). Thus,under the conditions used, the Donnan effect on the equilibrium distribution of diltiazem was negligible.

Diltiazem binding to human sera. Eight human sera were dialyzed against labeled diltiazem at an initial concentration of 100 ng/ml. Diltiazem was highly protein bound with a mean bound fraction of 0.81. There was significant inter-individual variation, with the highest free fraction of 0.24 being 1.6 times higher than the lowest free fraction of 0.15.

Diltiazem binding to purified protein fractions. At concentrations of protein ranging from 0.2 to 0.9 mg/ml, the relative binding of diltiazem to protein, defined as (bound/free)$\div$(protein,mg/ml) x100, was the following: LDL-129, HDL-102, AAG-53, VLDL-49, albumin-24,immunoglobulin (IgG)-22. LDL and HDL exhibited twice the relative binding of AAG and VLDL, and five times that of albumin. Stoichiometrically, binding to LDL is 150 times greater than that of albumin when the molecular weights are assumed to be $2x10^6$ amd $7x10^4$, respectively.

Diltiazem binding to LDL. Radiolabeled diltiazem-LDL complexes were treated with pronase to see if stripping of surface apoproteins would release ^{14}C-diltiazem into trichloroacetic acid (TCA) supernatant. After pronase digestion, diltiazem bound to surface apoproteins would become associated with TCA soluble materials whereas diltiazem bound to phospholipids or the neutral lipid core would remain TCA precipitable. The data in Table I show that the mean TCA

TABLE I. DISTRIBUTION OF ^{14}C-DILTIAZEM FOLLOWING PRONASE DIGESTION OF ^{14}C-DILTIAZEM-LDL COMPLEXES

	INCUBATION HOURS	TCA PRECIPITABLE RADIOACTIVITY, %	TCA SOLUBLE RADIOACTIVITY, %	ULTRA-CENTRIFUGATION "LDL" FRACTION
With	1	36	62	41
Pronase	3	48	51	53
Digestion	5	48	51	45
	7	43	55	48
	25	42	56	49
		(43.4 ± 5.0)	(55.0 ± 4.5)	(47.2 ± 4.5)
Without	1	50	48	42
Pronase	3	47	51	56
Digestion	5	49	50	41
	7	49	50	48
	25	46	53	44
		(48.2 ± 1.6)	(50.4 ± 1.8)	(46.2 ± 6.1)

precipitable radioactivity (43.4% ± 5.0) after pronase digestion was not significantly different from the mean (48.2% ± 1.6) without pronase digestion. Similarly, the mean TCA soluble radioactivity was not affected by pronase action on LDL (55.0 ± 4.5 vs. 50.4 ± 1.8). Pronase digestion also did not have any effect on the distribution of radioactivity in the "LDL fraction" obtained by ultracentrifugation (47.2% ± 4.5 vs. 46.2% ± 6.1). These data suggest that diltiazem binding to LDL is associated not with surface apoproteins but with either the phospholipids or the neutral lipid core, and that the binding is not freely reversible. Furthermore, binding rapidly reached equilibrium as there was no change in percent radioactivity beyond one hour of incubation.

DISCUSSION. Using a carefully validated three-chamber equilibrium dialysis system, we have confirmed that diltiazem at a therapeutic serum concentration of 100 ng/ml (3) is highly protein bound. We have extended the investigation to show that diltiazem binds not only to albumin, but also to LPs and AAG. In fact, the relative bindings of AAG and LP fractions are higher than that of albumin. The binding to LDL was sufficiently strong to survive ultracentrifugation in high salt medium as well as TCA treatment. There was negligible demonstrable binding to whole red cells but binding to red cell hemolysate was significant (15%) (unpublished data).

The finding that pronase digestion of LDL surface apoproteins did not release bound diltiazem suggests that diltiazem is not bound to the surface apoproteins. Whether diltiazem binds to the phospholipids or to the neutral lipid core is not clear. Although several drugs have been shown to bind to LPs(2), little is known about which component of LP is the binding site. Propranolol and tetracycline have been shown to bind to LPs in a way which suggests distribution to the lipid phase (9,10). Kristensen showed a positive correlation between imipramine binding and plasma apo B levels, although actual drug binding to apo B was not demonstrated (11). Drug binding to phospholipids would be novel and it will be interesting to see if diltiazem binds to the phospholipids of LDL. Experiments using phospholipase C to clarify this are in progress.

In the small group of patients tested for protein binding of diltiazem, there were large inter-individual varia-

tions in the extent of protein binding. The 1.6 fold variation in the free fraction (0.15-0.24) may be due to the highly variable plasma levels of the major diltiazem binders, LPs and AAG. Such variability has important therapeutic implications as hyperlipoproteinemia is common among patients on diltiazem therapy. Patients who are recovering from tissue injury such as myocardial infarction or coronary bypass surgery also can have markedly abnormal plasma AAG or LP levels because these proteins are acute phase reactants. A standard diltiazem regimen can lead to very different free drug levels and therapeutic effects. Thus, dosage adjustments to control drug levels within therapeutic concentrations will have to take into consideration the importance of diltiazem binding to AAG and LP.

The strong bindings of diltiazem to LP suggests that the concept of LP as transport molecules of lipids may need to be expanded to include the delivery of drug molecules from sites of absorption to sites of drug action, biotransformation and elimination. At the level of mechanism of drug-cell interaction and internalization of CABs, it is tempting to speculate that, given the strong binding to LDL, a mechanism of cellular uptake of some of the CABs may be mediated through cellular receptors for LPs, such as those for LDL.

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