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Binding of d-*Tubocurarine to Plasma Proteins in Normal Man and in Patients with Hepatic or Renal Disease*

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The binding of *d*-tubocurarine to plasma proteins in healthy adults and in patients with hepatic or renal disease was investigated by equilibrium dialysis and gel filtration using tritium-labeled drug. In healthy individuals a mean of 44.4 per cent of the drug was bound at a concentration of 5 μg/ml and 39.9 per cent at 50 μg/ml, as measured by equilibrium dialysis. Gel filtration gave results suggesting lower binding, consistent with dissociation of the drug–protein complex during the column separations. There was no significant difference, by these techniques, in the proportions of *d*-tubocurarine binding to plasma proteins of healthy patients as compared with patients with hepatic or renal disease. (Key words: Plasma protein binding; *d*-Tubocurarine; Liver disease; Kidney disease.)

WITHIN THE VASCULAR COMPARTMENT, a variable portion of a drug is reversibly bound to plasma proteins; however, only the free or unbound fraction is diffusible and pharmacologically active. The protein-bound drug molecules are hindered from gaining access to specific receptors in the tissues and to sites of metabolism and excretion. The influence of plasma binding on the pharmacokinetics of drug action and interaction has been reviewed in several recent publications.[1-3]

Data about the binding of drugs used in anesthesia practice are relatively scanty, particularly in the case of the myoneural blocking agents. There is wide individual variation in the responses to the latter drugs, which has been attributed by some investigators to differences in the extents of protein binding.[4] Dundee and Gray,[5] and later Haselhuhn,[6] reported increased resistance to *d*-tubocurarine in patients with dysfunction of the liver. The same was found in patients with bilharzial cirrhosis of the liver by El-Hakim and Baraka.[7] To test these findings, Payne and Webb [8] studied the effects of normal serum and serum from patients with jaundice or hepatic disease on *d*-tubocurarine's antagonism of acetylcholine-induced contracture of the frog rectus abdominis muscle. They found decreased action of the myoneural blocker in the presence of serum from jaundiced patients, which they suggested might be due to its increased protein binding. Stout,[9] Baraka *et al.*,[10] and Stovner *et al.*[11] independently reported that *d*-tubocurarine requirements in patients during surgery correlated directly with the plasma globulin level; the higher the latter, the larger the dose of *d*-tubocurarine needed. This seemed to agree with the decreased sensitivity to the drug of patients with hepatic disease, in whom inversion of the albumin/globulin ration is common in the chronic active states of the disease.

We have attempted to study interindividual differences in the plasma protein binding of *d*-tubocurarine. We examined the protein binding of the drug in plasma of patients with hepatic and renal disease. The latter were included because of evidence of decreased binding of some drugs in uremic patients and in anephric patients on dialysis. The effect of temperature on the extent of binding was also examined because of the known decreased sensitivity of the neuromuscular junction to *d*-tubocurarine at low temperatures.

The plasma protein binding of the ^3H-labeled drug was determined by equilibrium dialysis and gel filtration.

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Methods

Subjects

The normal group studied consisted of healthy human volunteers taking no medication. The patients with hepatic disease, except for one patient who had viral hepatitis, had moderate to severe hepatic insufficiency resulting from cirrhosis, confirmed by liver biopsy. The third group was composed of patients with chronic renal failure undergoing regular hemodialysis. No patient received any medication for at least 12 hours prior to collection of the sample. None received a blood transfusion for four weeks prior to collection of the sample.

Equilibrium Dialysis

Blood was collected in heparinized tubes † between 8 and 9 AM, except that a second sample was obtained from patients undergoing renal dialysis after dialysis. Plasma was separated from the cells by centrifuging at 1,500 rpm for 15 minutes and was then removed and stored at 4 C. Binding studies were carried out within 24 hours of collection of the sample. A plasma sample, 0.9 ml, was mixed with 0.1 ml of unlabeled *d*-tubocurarine solution and 10 μl of *d*-tubocurarine-^{3}H solution.‡ The unlabeled drug solutions contained either 50 μg/ml or 500 μg/ml, while the labeled drug solution contained 20 μg/ml dissolved in Sørensen's phosphate buffer, *p*H 7.4. The mixture was incubated for 15 minutes at 25 C before transfer to one side of a plexiglass equilibrium dialysis cell.§ The other side was filled with 1.0 ml of Sørensen's phosphate buffer, *p*H 7.4. Each side contained a small stirring magnet, and the two sides were separated by a presoaked cellulose dialysis membrane.¶

† Heparinized tubes: Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey.

‡ Exchange tritiation of *d*-tubocurarine chloride was performed by New England Nuclear Corporation, Boston, Massachusetts. Specific activity was 6.9 mCi/mg. The crude tritiated mixture was purified by partitioning between ethylene dichloride and potassium iodide–glycine buffer. The radiochemical purity was established using thin-layer chromatography.[19]

§ Bel-Art, Pequannock, New Jersey.

¶ Size 1.125 inch inflated diameter, pore size 4.8 mμ. Fisher Scientific Co., Chicago, Illinois.

Table 1. The Plasma Protein Binding and Electrophoretic Patterns in Normal Subjects and in Patients with Hepatic or Renal Disease*

	Number	Per Cent Binding at 5 μg/ml	Per Cent Binding at 50 μg/ml	Total Protein Concentration (g/100 ml)	Albumin (g/100 ml)	α_1-Globulin (g/100 ml)	α_2-Globulin (g/100 ml)	β-Globulin (g/100 ml)	γ-Globulin (g/100 ml)
Normal subjects	5	44.4 ± 7.7	39.9 ± 3.1	7.6 ± 0.4	3.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	1.2 ± 0.1	1.8 ± 0.3
Patients with hepatic disease	8	36.8 ± 5.3	37.1 ± 8.5	7.6 ± 1.2	2.4 ± 0.9	0.3 ± 0.04	0.7 ± 0.2	1.1 ± 0.8	4.4 ± 1.11
Patients with renal disease	6	41.2 ± 3.4	42.4 ± 0.3	7.0 ± 0.7	3.2 ± 0.3	0.4 ± 0.06	0.8 ± 0.1	1.4 ± 0.3	1.1 ± 0.4

* Values are expressed as means ± SD. Means were compared by analysis of variance.

† The gamma-globulin in patients with hepatic disease is the only value significantly different from values in normal subjects and renal disease patients.

TABLE 2. Effects of Temperature on the Binding of *d*-Tubocurarine

	Per Cent Bound at Temperature		
	4 C	26 C	37 C
Subject 1	33.3	30.1	26.5
Subject 2	37.3	35.9	30.2
Subject 3	35.9	28.4	25.8

TABLE 3. Percentages of *d*-Tubocurarine Bound at a Concentration of 50 μg/ml Using Equilibrium Dialysis and Gel Filtration

	Per Cent *d*-Tubocurarine Bound at 50 μg/ml	
	Equilibrium Dialysis	Gel Filtration
Subject 4	40.6	38.8
Subject 5	48.7	31.8
Subject 6	37.0	31.3

Dialysis was carried out for 6 hours at 4 C with constant slow stirring. For three subjects, dialysis was also conducted at 26 and 37 C.

Preliminary experiments were conducted to establish the time needed to reach equilibration, to study the possibility of adherence of the drug to the dialysis membrane, and to determine any interaction between *d*-tubocurarine and the trace of heparin in the plasma. All experiments were carried out at least in duplicate.

GEL FILTRATION

Sephadex G-25/medium grade ** or Bio Gel P-2,†† which was used later, was allowed to swell for at least 6 hours in Sørensen's phosphate buffer, *p*H 7.4, and packed in a glass tube plugged loosely with glass wool to produce a column 43 × 0.3 cm. A minimum volume of 25 ml of the buffer solution was passed through the packed column to insure proper settling of the gel. A plasma–drug mixture was prepared as in the equilibrium dialysis method, using a final concentration of 50 μg/ml *d*-tubocurarine. A 0.2-ml fraction of the mixture was applied to the column and was followed by a small volume of buffer to rinse the protein–drug complex from the sides of the tube. The mixture was eluted with buffer by gravity and fractions were collected with a Buchler Fractomat fraction collector. The first ten fractions containing the protein were 0.5 ml each, while subsequent fractions (protein-free) were 1 ml each. Protein concentration in the fractions was determined by the biuret reaction [12] and measured in a Gilford Model 300 micro-sample spectrophotometer. All experiments were conducted at room temperature.

QUANTITATION

In the radioassay, a 0.1-ml sample was pipetted into a 20-ml liquid scintillation vial which contained 0.4 ml of NCS solubilizer.†† Solution was aided by warming the vial slightly. After solution was complete, 10 ml of Bray's solution §§ were added. Samples were counted after allowing sufficient time to give an error of less than 2 per cent. Counting was done initially on a Beckman LS-100 Soft Beta Spectrometer, whereas later counts were made on a Packard 2420 Liquid Scintillation Counter. Plasma samples from the patients with hepatic disease, especially those containing high bilirubin levels, gave low counts, possibly due to the quenching effect of the pigment. Samples were counted a second time after 24 hours, by which time the effect of the yellow color had diminished. Initial counts from patients undergoing renal dialysis were much higher than was reasonable from the standpoint of material balance of the isotope added. We did not specifically determine the cause, since when second counts were made after 24 hours the fluorescence-enhancing material had apparently decayed.

The concentration of the plasma protein fractions was determined by electrophoresis in the clinical chemistry laboratories of Iowa University Hospitals.

** Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

†† Bio-Rad, Richmond, California.

†† Amersham/Searle Corporation, Arlington Heights, Illinois.

§§ Naphthalene 60 g, PPO 4 g, POPOP 0.2 g, ethylene glycol 20 ml, ethanol 100 ml, and dioxane to make one liter.

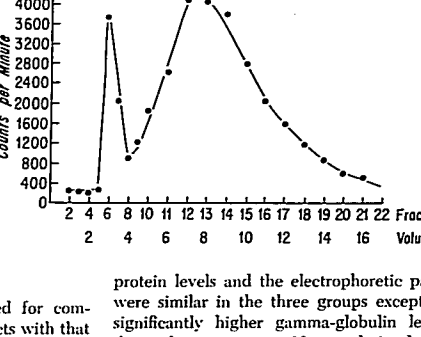

FIG. 1. Gel-filtration of ^{3}H-labeled *d*-tubocurarine added to plasma at a final concentration of 50 μg/ml and eluted with Sørensen buffer, pH 7.4, 0.5 molar. Samples from 2 to 10 are 0.5 ml and from 11 to 22, 1 ml. A Bio-Gel P-2 43 × 0.3 cm column was used at room temperature.

STATISTICAL ANALYSIS

Analysis of variance [12] was used for comparing the binding of normal subjects with that of diseased patients. The associations between binding and total protein and electrophoretic pattern were estimated by linear correlation coefficients.[14] The paired t-test was used for comparison of pre- and post-hemodialysis samples. $P < 0.05$ was considered significant.

Results

EQUILIBRIUM DIALYSIS

The extent of binding was estimated after analysis of the drug in the two sides of the dialysis cell. Since at equilibrium the concentrations of the free drug were the same in the two compartments, the difference between concentrations of the drug on the two sides represented the bound fraction. Concentrations were measured in terms of counts per minute (cpm) and expressed as percentages of total drug concentration.

Preliminary experiments demonstrated that 6 hours was sufficient time for the unbound drug on the two sides of the membrane to attain equilibrium. The membrane did not absorb any significant amount of the drug at the two concentrations studied. Heparin did not affect binding, as similar results were obtained using either serum or plasma.

In normal individuals, 44.4 ± 7.7 per cent of the drug was bound at the 5 μg/ml concentration and 39.9 ± 3.1 per cent at the 50 μg/ml concentration. There was no significant difference between binding in this group and binding in patients with hepatic or renal disease, as shown in table 1. The total plasma protein levels and the electrophoretic patterns were similar in the three groups except for a significantly higher gamma-globulin level in the cirrhotic patients. No correlation between the extent of binding and any of the plasma protein fractions could be found. Interindividual variability was relatively large regardless of whether the individuals were normal or diseased. The method had a standard deviation of only 1.2 (this was reached by dialyzing the same sample simultaneously in five different cells).

There was a progressive decrease in binding as the temperature was raised from 4 C to 26 C and then to 37 C, as shown in table 2.

GEL FILTRATION

Results from the Sephadex column were erratic. The highest counts were obtained from the first fraction collected, but this contained neither protein nor *d*-tubocurarine when assayed spectrophotometrically. We therefore replaced Sephadex with Bio Gel. The drug was eluted in two peaks, a sharply defined narrow peak associated with the drug–protein complex, followed by a broader one associated with the free drug fraction (fig. 1). Resolution of the peaks was not complete, possibly indicating some dissociation of the drug–macromolecule complex occurring during the elution with buffer. The percentage of bound drug was determined by comparing the amount associated with the protein peak (bound drug) with the total amount of drug (bound and free). The extent of binding was always less than that obtained by equilibrium dialysis (table 3).

Discussion

We chose equilibrium dialysis as the main technique for studying the plasma protein binding. Like the other available methods, it could be open to several sources of error. Equilibrium will not be attained in the specified time if the membrane is not freely permeable to the drug, if stirring is not effective, if the temperature is not constant, etc. We tried to avoid these errors, by soaking the membrane before use, applying constant stirring at a constant temperature, and demonstrating that the membrane did not absorb the drug. Dialysis was carried out at 4 C, rather than at body temperature, in order to avoid the possibility of bacterial contamination and deterioration of the plasma. *d*-Tubocurarine is a quaternary ammonium base which may combine with the strongly acidic groups of heparin to form a poorly diffusible complex. It is known that heparin in high doses, 50–100 mg/kg, can antagonize the neuromuscular blocking action of *d*-tubocurarine *in vivo*.[15] However, our finding that similar binding could be obtained with serum as well as plasma suggests that no significant interaction occurred at the concentrations used. Cohen *et al.*[16] studied the binding of *d*-tubocurarine by normal plasma. Using a concentration of 20 μg/ml *d*-tubocurarine, they reported that approximately a third of the drug was protein-bound. Our results approximate their findings. Dimethyl *d*-tubocurarine is apparently more extensively bound, since Dal Santo[17] reported 70 per cent binding using an ultrafiltration method.

Gel filtration technique was used to compare the results with values obtained by equilibrium dialysis. Results obtained with the Sephadex column suggested an exchange of hydrogen from Sephadex with tritium of the labeled drug, and therefore Bio Gel was used in its place. We reasoned that since the latter is a polyacrylamide resin, no hydrogen exchange would take place. The extent of binding was less than that measured by equilibrium dialysis, which may be explained by some dissociation of the bound drug and the higher temperature at which the experiment was conducted. We did not attempt to quantitate the difference between the two techniques, but rather examined the direction of the difference.

There was some variability in binding among different individuals. Cohen and his colleagues found a poor correlation between the total plasma concentration of *d*-tubocurarine and its clinical effect.[15] Katz was not able to predict the neuromuscular blocking effect at any dosage schedule.[19] It is tempting to speculate that the interindividual differences in the extents of binding may at least partly explain this large variability in response. A study which involves correlating the drug concentration in the plasma, in terms of "total" and "free," with the response curves for individual subjects could clarify this point.

There was a progressive decrease in binding with increasing temperature, a feature quite characteristic of non-covalent forms of binding. The data, however, were not adequate for quantitative measurement. It has been established that hypothermia reduces myoneural blockade produced by *d*-tubocurarine.[20] One reason may be the increased protein binding; as a result, less drug becomes available to the receptor sites at the neuromuscular junction. Other possible causes to consider are decreased cholinesterase activity, alterations in the reaction of the drug with the receptor sites, or variations in membrane potential and conductance.[21]

The effect of disease on the plasma protein binding of drugs has recently started to attract the attention of investigators. It was found that the binding of some acidic drugs, such as sulfonamides[22] and diphenylhydantoin,[23] was decreased in uremic plasma. The impairment of binding correlated strongly with the degree of azotemia, but correlated weakly with the concentration of serum proteins. However, binding of the basic drugs which have been tested so far in patients with uremia appears to be normal. Our findings with *d*-tubocurarine add another example. Basic drugs may have binding sites different from those of acidic drugs and may not be affected in the same way.

Dundee and Gray, Haselhuhn, and El-Hakim and Baraka independently reported decreased sensitivity to *d*-tubocurarine in patients with hepatic disease. These reports were later linked with other reports by Stout, Baraka and Gabali, and Stovner, Theodorsen and Bjelke, demonstrating a correlation between the plasma

globulin level and *d*-tubocurarine requirements of patients. However, Cohen and colleagues [24] reported that *d*-tubocurarine was bound equally by the individual protein fractions tested, albumin, gamma-globulin and fibrinogen. Our study demonstrated no change in binding in patients with hepatic disease. These patients, while showing increased gamma-globulin levels, did not differ in total plasma protein concentrations from the other two groups studied. It should be noted that we used relatively small groups, and it can be argued that failure to show a statistically significant difference may not mean conclusively that none exists. However, the trend in patients with hepatic disease was towards less binding, and we suspect that if more measurements had demonstrated a difference, it would have been in the direction of deficient rather than enhanced binding.

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