BINDING OF TUBOCURARINE TO SPECIFIC SERUM PROTEIN FRACTIONS

M. M. GHONEIM AND H. PANDYA

SUMMARY

The binding of tritium-labelled tubocurarine to separate protein fractions of sera from normal subjects and patients with hepatic disease was measured by an electrophoretic technique. The binding of the drug to gamma globulin and albumin solutions also was studied using equilibrium dialysis. The major portion of the drug, 82-90%, was bound to the gamma globulin electrophoretic fraction of both normal and patient sera. However, as measured by equilibrium dialysis, only 15.8% of the drug was bound to the gamma globulin and 23.8% to albumin. Equilibrium dialysis yields the data by which the validity of other methods for measuring drug-protein binding can be judged. Since in a previous study we also found no difference in binding of the drug between healthy individuals and patients with hepatic disease, equilibrium dialysis measurements may reflect better the binding of the drug in vivo.

It has long been recognized that the binding of drugs to plasma proteins may alter markedly the magnitude and duration of their pharmacological response. An increase in binding of tubocurarine decreases the blockade produced by a given dose (Baraka and Gabali, 1968) and it may also slow the rate of its elimination from the body (Eger, 1974). In 1958, Aladjemoff, Dikstein and Shafrir reported that in most individuals the major portion of tubocurarine was bound to the gamma globulin fraction of the plasma proteins. This work was referred to frequently over the years and had been cited as an explanation for an alleged resistance to tubocurarine by patients with hepatic disease (Dundee and Gray, 1957). A correlation between the drug requirement of patients and their albumin/globulin ratios was explained similarly (Baraka and Gabali, 1968; Stovner, Theodorsen and Bjelke, 1971). On the other hand, Ghoneim and his colleagues in 1973 found no difference in binding of the drug between healthy individuals and patients with hepatic disease. There was a tendency towards a lesser degree of binding in the latter, but because of the small number of subjects studied, the difference was not statistically significant. This was confirmed later by Reidenberg and Affrime (1973) who indicated, in a preliminary report, that patients with hepatic cirrhosis have some impairment to the binding of drugs.

There is obviously a controversy about the specific plasma protein fraction which binds tubocurarine. If the drug is bound mostly to gamma globulin, patients with cirrhotic disease of the liver should bind the drug in plasma more than healthy subjects. Aladjemoff's work (1958) was carried out by using an electrophoretic technique, while ours employed equilibrium dialysis. Therefore we planned the present study to investigate whether we could duplicate the results of Aladjemoff and her co-workers. Since hepatic disease may affect the pattern of binding to individual fractions, cirrhotic patients were included in the study. Electrophoresis, in the way it is used commonly, does not give a precise quantitative estimate of the degree of binding. Therefore, we elected to confirm our results by measuring the amount of drug bound to separate protein fractions by equilibrium dialysis.

METHODS

Materials
d-Tubocurarine-3H. Tritiated tubocurarine was obtained from the New England Nuclear Corporation, Boston, Massachusetts. Its specific activity was 6.9 mCi/mg. Purification was achieved by thin layer chromatography (Skivington, 1972).

Serum. Blood was obtained from eight healthy male volunteers who were not taking any medication. Blood was obtained also from nine male patients suffering from hepatic cirrhosis, as confirmed by liver biopsy. If they were receiving drugs, they abstained...
for at least 12 hr before the collection of the sample. None of the patients received a blood transfusion in the 2-week period before the study. Clotted blood was centrifuged and the resulting fresh serum was used.

Protein fractions. Crystalline human plasma albumin and human gamma globulin, fraction II, were purchased from Miles Laboratories, Kankakee, Illinois. We prepared also gamma globulin from 200 ml of plasma obtained from a healthy volunteer. Gamma globulin was precipitated by adding a saturated solution of ammonium sulphate to make 45% of the plasma volume. Centrifugation was then carried out at 15,000 rev/min at 4°C for 15 min, and the precipitate was suspended in phosphate buffer (pH 7.4). The solution was then dialysed against phosphate buffer at 4°C for 3 hr. After dialysis it was reprecipitated again with ammonium sulphate and the process was repeated four times. After the fourth precipitation the sample was dialysed for 24 hr at 4°C and the concentration of gamma globulin was assayed spectrophotometrically.

Binding studies

Electrophoresis. A Beckman microzone apparatus was used (Beckman Instruments Inc., 2500 Harbour Blvd, Fullerton, California). Its acetate membrane, designed to take eight samples simultaneously, was placed in veronal buffer (pH 8.6) inside the apparatus. A 25-μlitre portion of the alcoholic solution of d-tubocurarine-4H was evaporated and the residue was redissolved in 50 μlitrre of serum. Seven samples, 0.25 μlitre each, of the serum-drug mixture were then applied to the membrane beside a pure serum sample. A 220-V current was passed for 40 min. At the end, a strip of the membrane, corresponding to the position of the pure serum sample, was cut lengthwise and stained to show the protein bands. It was then realigned with the rest of the membrane and strips were cut from the latter corresponding to each protein fraction. Each strip was placed in a liquid scintillation vial and 10 ml of Bray's solution* was added. The vial was shaken gently until the membrane dissolved. Radioactivity was measured in a Beckman liquid scintillation spectrometer. Quenching was monitored with the external standard.

Equilibrium dialysis. Tubocurarine was added to a gamma globulin or albumin solution to produce a concentration of 5 μg/ml in one side of an equilibrium dialysis cell. The other side was filled with a similar volume of Sørensen's phosphate buffer (pH 7.4). The two sides were separated by a pre-soaked cellulose dialysis membrane. Gamma globulin was used in a concentration of 2.0 g/100 ml and albumin in a 4% solution. Experiments with gamma globulin were performed also at pH of 8.6. The dialysis system was maintained at 4°C with continuous stirring. After 6 hr, sufficient time for equilibration, duplicate 0.2-ml samples from both sides of the cell were pipetted into liquid scintillation vials and the same technique was used for measuring radioactivity as was described before, except that an NCS solubilizer (Amersham/Searle Corporation, Arlington Heights, Illinois) was added (0.4 ml vial). Leakage of protein through the dialysis membrane was checked by precipitation test with trichloroacetic acid and the biuret reaction.

The concentration of tubocurarine determined in the buffer side represented the free drug concentration, while its concentration in the protein side represented the sum of free and bound drug. Therefore:

\[
\text{Fraction bound} = \frac{\text{amount in protein side} - \text{amount in buffer side}}{\text{total amount}}
\]

RESULTS

Electrophoresis. There was a tendency for lower albumin and higher gamma globulin concentrations in patients as compared with healthy subjects, but the difference was not statistically significant (table I). The major portion of tubocurarine, 82–90%, was bound to gamma globulin, with the remaining part being associated with beta globulin, albumin and the rest of the fractions (table II). The pattern of binding was the same in normal subjects as in patients with liver disease.

| Table I. Serum electrophoretic patterns of normal subjects and patients with hepatic disease. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Protein Fraction | Normal subjects | Patients with hepatic disease* | Normal subjects | Patients with hepatic disease* |
| Albumin | 57.72 ± 5.37 | 57.72 ± 5.37 | 57.72 ± 5.37 | 57.72 ± 5.37 |
| α₁-Globulin | 5.80 ± 1.11 | 5.80 ± 1.11 | 5.80 ± 1.11 | 5.80 ± 1.11 |
| α₂-Globulin | 10.10 ± 0.94 | 10.10 ± 0.94 | 10.10 ± 0.94 | 10.10 ± 0.94 |
| β-Globulin | 17.21 ± 1.98 | 17.21 ± 1.98 | 17.21 ± 1.98 | 17.21 ± 1.98 |
| γ-Globulin | 18.12 ± 2.62 | 18.12 ± 2.62 | 18.12 ± 2.62 | 18.12 ± 2.62 |

Each value is expressed as per cent of the total protein. †Mean values of serum samples from eight subjects. ‡Mean values of serum samples from nine patients. *SEM.
**Table II.** Radioactivity (c.p.m.) attached to protein fractions after electrophoresis.

<table>
<thead>
<tr>
<th></th>
<th>Albumin</th>
<th>$\alpha_1$-Globulin</th>
<th>$\alpha_2$-Globulin</th>
<th>$\beta$-Globulin</th>
<th>$\gamma$-Globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>5.17 ± 0.76</td>
<td>3.21 ± 0.47</td>
<td>3.80 ± 0.63</td>
<td>5.59 ± 0.95</td>
<td>81.87 ± 1.78</td>
</tr>
<tr>
<td>Patients with hepatic disease</td>
<td>3.34 ± 0.73</td>
<td>2.23 ± 0.28</td>
<td>2.27 ± 0.41</td>
<td>2.68 ± 0.57</td>
<td>89.89 ± 1.66</td>
</tr>
</tbody>
</table>

Each value is expressed as per cent of the total count attached to proteins. †Mean values of serum samples from eight subjects. ‡Mean values of serum samples from nine patients. *SEM.

**Equilibrium dialysis.** The gamma globulins from the commercial manufacturer and that which we prepared gave identical binding values (15.8%). There was no significant difference between the binding of 4% albumin and 2% gamma globulin. Changing the pH of the dialysis medium from 7.4 to 8.6 did not increase the binding capacity significantly (table III).

**Table III.** Binding of tubocurarine to albumin and gamma globulin fractions as determined by equilibrium dialysis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tubocurarine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma globulin 2%</td>
<td>15.82 ± 0.34</td>
</tr>
<tr>
<td>Albumin 4%</td>
<td>23.79 ± 0.72</td>
</tr>
<tr>
<td>Gamma globulin 2% at pH 8.6</td>
<td>16.55 ± 0.22</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of five determinations.

**DISCUSSION**

It is important to determine the protein fractions with which tubocurarine is bound, because it may influence the dose-response relationship of the drug in some disease states, such as hepatic cirrhosis, hypoalbuminaemia and hypogammaglobinaemia. The most important contribution to drug binding is that of albumin. Most acidic drugs bind to it exclusively, whereas some of the basic drugs bind to other proteins also. The antibody gamma globulins interact very specifically with other antigens, but to a negligible extent with most drugs (Goldstein, Aronow and Kalman, 1968). Nevertheless, by using electrophoresis, tubocurarine was found to be bound most by gamma globulin and least by albumin and the other globulins. This pattern of binding proved to be very similar to that of dimethyltubocurarine (Skivington, 1972). The pattern of binding in patients with hepatic disease was similar to that of healthy individuals. We did not encounter a case where the pattern of binding was changed as reported by Aladjemoff and others (1958). They cited two patients who were refractory to tubocurarine and in whom the major portion of the drug moved with the albumin fraction.

Equilibrium dialysis yielded results which were unexpected in view of those of the electrophoretic technique. Only 15.8% of tubocurarine was bound to gamma globulin at its physiological concentration, and a similar percentage of the drug was bound to albumin. The conditions under which binding was studied with both techniques differed in at least two respects. The first was pH. While equilibrium dialysis was carried out at pH 7.4, electrophoresis was performed at pH 8.6 to achieve separation of the various protein fractions. When we repeated the dialysis experiment at pH 8.6 the binding was almost similar. The second factor was temperature. Dialysis experiments were conducted at 4°C, while that of electrophoresis was much greater. We do not know the exact temperature inside the apparatus during the passage of electric current, but obviously it would be greater than room temperature. In previous work we demonstrated a progressive decrease in binding as the temperature was increased from 4°C to 26°C and then to 37°C (Ghoneim et al., 1973). This would not support the lower degree of binding obtained by equilibrium dialysis. One may speculate about the unbinding of protein-drug complexes with low affinity during electrophoresis, while those with higher affinity would stay intact. This would be similar to the case of binding of salicylic and acetylsalicylic acids (Kramer and Routh, 1973). In spite of high binding (96%) of salicylic acid by equilibrium dialysis, very low binding values were obtained by gel filtration, indicating extensive unbinding of the weak albumin-salicylic acid complex. The binding sites of acetylsalicylic acid were by contrast much stronger, leading to recovery, from the column, of most of the drug in the bound form. Dilution of the serum by the buffer and the effect of the electric current may promote uncoupling of weak complexes in the same manner as the gel filtration column. If that is the case, one may conclude that the bonds between tubocurarine and albumin binding sites are characterized by weak forces which are easily disrupted, while those between the drug and gamma globulin are much stronger.

Equilibrium dialysis yields the data by which the validity of other methods for estimating the degree of binding of drug molecules by proteins can be judged (Bush and Alvin, 1973) and probably gives the best...
measure of binding capacity (McArthur and Smith, 1969). Aladjemoff and her colleagues (1958) reported an interesting experiment in dogs. They observed that when tubocurarine which was pre-incubated with albumin was injected, there was no pharmacological response. However, when the same dose was pre-incubated with gamma globulin, paralysis occurred although it was of smaller magnitude than that following injection of the drug pre-incubated with normal saline. These results did not agree with their electrophoretic data, which suggested that the drug was bound most by gamma globulin and least by albumin. These reasons, combined with our previous study of normal subjects and patients with hepatic disease, tend to confirm our belief that the results of equilibrium dialysis may reflect better the binding in vivo.

ACKNOWLEDGEMENTS

We are indebted to Dr A. A. Spector, Associate Professor of Biochemistry and Internal Medicine, College of Medicine, for his valuable advice. Dr C. Vestling, Professor and Chairman of the Department of Biochemistry kindly gave us permission to use equipment in his laboratory.

REFERENCES


