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The binding of physostigmine to human serum albumin

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Abstract—The binding of [^{3}H]physostigmine to crystallized human serum albumin (HSA) has been investigated using equilibrium dialysis. The percentage bound to 1% (w/v) HSA decreased from 18 to 4% as the total concentration of physostigmine increased from 3·3 nM to 2·7 μM (0·9 to 750 ng mL^{-1}). A single class of specific binding sites with a large affinity constant, $K = 8 \times 10^7$ L mol^{-1}, was identified. The concentration of binding sites was approximately 3 nM. The Michaelis constants for human serum cholinesterase and albumin were the same; an explanation for these results is that the drug is binding to a trace cholinesterase, in the albumin.

The binding of physostigmine to rat and human plasma and to crystalline rat and human serum albumins has been studied by Unni & Somani (1985) using ultrafiltration. Those workers ignored the fact that at low concentrations physostigmine is rapidly metabolized by human plasma (Whelpton 1983; Whelpton & Moore 1985). This may explain the shape of their percent bound versus concentration plot for human plasma and why the slope of the Scatchard plot was positive. With crystalline proteins they obtained relatively high equilibrium constants and what appear to be very small numbers of molecules bound per mol of protein. Using a different technique (equilibrium dialysis) we present figures for the binding of physostigmine to purified human albumin for which there appears to be a very high affinity site of very low binding capacity.

Materials and methods

Materials. Physostigmine, human serum albumin (HSA) Fraction V (Lot 16F-9344), α_1-acid glycoprotein and butyryl cholinesterase, BChE (from Sigma, Poole, Dorset, UK) were used without further treatment. [^{3}H]Physostigmine (16·8 Ci mmol^{-1}) prepared by catalytic reduction of the bromo derivative, was custom synthesized by Amersham International (Amersham, UK). Cocktail W scintillation fluid was purchased from BDH (Poole, Dorset, UK).

Equilibrium dialysis. HSA solutions (1% w/v) were prepared in 0·1 M phosphate buffer, pH 7·4. Lengths (approximately 10 cm) of Visking tubing, 1 cm wide when flat, were soaked in buffer until supple. Excess water was removed with a tissue and one end closed with a double knot. HSA solution (0·5 mL) was introduced and the open end closed with a further double knot such that an air bubble remained in the sac to aid mixing. The excess tubing was trimmed off and the outsides of the sacs rinsed with buffer before the sacs were added to tubes containing physostigmine solutions (2 mL) in the range 1 – 1000 ng mL^{-1}. The capped tubes were shaken mechanically at room temperature (20 ± 1°C) for 18 h. The sacs were opened and duplicate samples (0·2 mL) taken from inside and outside the sacs for scintillation counting. Quench corrections were made using the external standard method.

Control experiments were performed using buffer solution in place of albumin solution. The purity of the physostigmine before and after dialysis was determined by chromatographing the samples on a cyanopropyl HPLC column and collecting fractions for scintillation counting (Hurst & Whelpton 1989).

Binding to 0·05% (w/v) α_1-acid glycoprotein was determined by equilibrium dialysis as described above using an initial physostigmine concentration of 50 ng mL^{-1}.

Cholinesterase activities. The cholinesterase activities of 1% HSA and BChE (0·1 Sigma unit mL^{-1}) were determined using a modified method of the colorimetric method of Ellman et al (1961) as described previously (Whelpton 1978) using butyrylthiocholine as substrate.

Data analysis. The binding constant, K, was obtained using the modified Scatchard plot of Rosenthal (1967) in which C_b/C_f is plotted against C_b. The graphic solution was performed on a microcomputer using a specially written GRAFIT program (Whelpton 1988). The data were entered as concentration inside the sac (C_t) versus the concentration outside (C_f). The concentration bound, C_b, was obtained from the difference. The data were fitted statistically using the LIG1 program supplied with MKMODEL (Biosoft, Cambridge, UK) to find the dissociation constant, K_d, and B_{max}.

Michaelis constants and V_{max} values were determined from an iterative fit of the data to a Lineweaver-Burk plot using the program described by Nielsen-Kudsk (1983).

Results

The fraction of physostigmine bound decreased with increasing physostigmine concentrations. At a total concentration of 0·9 ng mL^{-1} the mean percent bound, ±s.d., was 17·8 ± 3·5 (n = 4). When the total concentration was 750 ng mL^{-1}, the corresponding figure was 4·2 ± 1·6. However, the control experiment gave a mean value of 3·1% bound (range 1·9 ± 1·0 to 3·9 ± 0·9, n = 10) for all concentrations of physostigmine in the absence of albumin. There was no binding to α_1-acid glycoprotein.

The curved Rosenthal plot was resolved into two components (Fig. 1). The shallow part corresponded to the non-specific binding demonstrated in the control experiment. The slope of the steep component gave a binding constant of $8 \cdot 1 \times 10^7$ L mol^{-1}. The capacity for this site was 2·9 nM from which the number of molecules of physostigmine bound per protein molecule, N, is 2×10^{-5}. These values were in reasonable agreement with those of a pilot experiment ($K = 8 \cdot 6 \times 10^7$ L mol^{-1}, $N = 8 \times 10^{-6}$) using only 4 initial concentrations.

The statistical plot of bound concentration versus free concentration gave $K_d = 12 \cdot 6 \pm 4 \cdot 4$ nM and $B_{max} = 2 \cdot 8 \pm 0 \cdot 6$ nM. These values are in agreement with those from the Rosenthal plot, the reciprocal of K_d being $7 \cdot 9 \times 10^7$. Non-specific binding was estimated as 4·2 ± 0·3%.

Comparison of the HPLC before and after binding (Fig. 2) showed only slight degradation of physostigmine during the course of the experiment (from 98% to 96·4%). The major radioactive impurity in the original chromatographed with the same retention volume as [^{3}H]water (Hurst & Whelpton 1989) whereas there are clearly additional peaks with retention times

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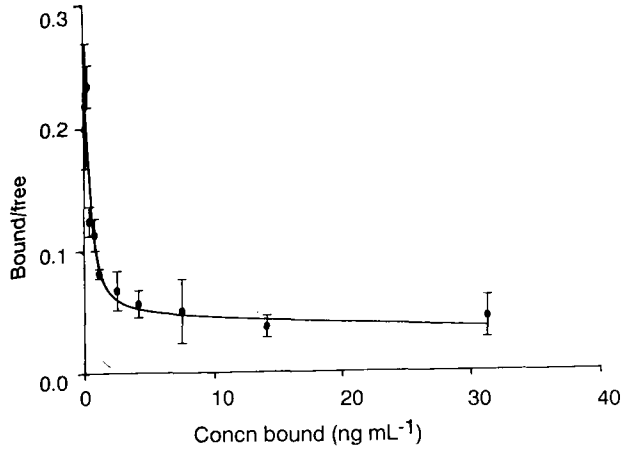

FIG. 1. Modified Scatchard plot for binding of physostigmine to human serum albumin. The solid line was obtained by fitting the data graphically to a model with two classes of binding sites. Each point is the mean of 4 determinations ± s.d.

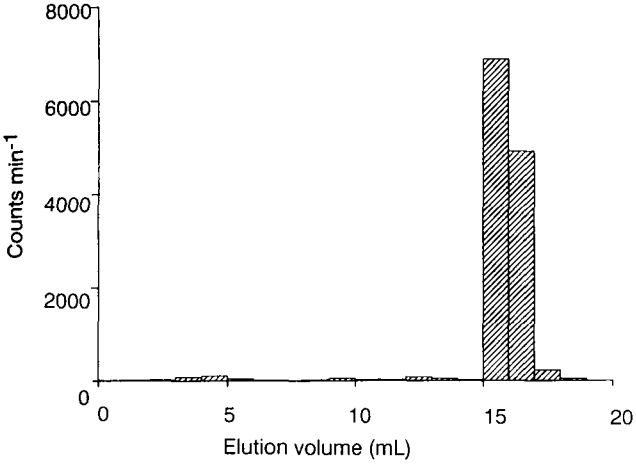

FIG. 2. Radioactivity in fractions of HPLC eluent collected to check purity of sample after equilibrium dialysis.

equivalent to the hydrolysis product eseroline and its oxidation product, rubreserine.

The K_m values (±s.d.) for butyryl cholinesterase using butyrylthiocholine as substrate was 27 ± 3 μM (n = 6) and for HSA 25 ± 2 μM (n = 8). Comparing the V_{max} values, 1 mL of 1% HSA was equivalent to 0·08 units of cholinesterase.

Discussion

Our binding constant was approximately 100 times greater than that determined by Unni & Somani (1985) and the number of binding sites per molecule of protein was correspondingly lower. It is difficult to rationalize why physostigmine should bind to only one albumin molecule in every 50 000 unless it is assumed that the drug is binding to another macromolecule present in only trace amounts. The high affinity of binding is in keeping with the macromolecule being an enzyme, possibly cholinesterase, as this is known to be present in Cohn Fraction V (Documenta Geigy 1962). This is supported by the similarity in K_m values obtained for HSA and human serum cholinesterase. Our value for BChE is somewhat higher than those of Rush et al (1985) 20 μM and Wetherell & French (1986), 17 μM. However, there was no significant difference between the Michaelis constants for HSA and BChE when determined under the same conditions.

The Michaelis constant for the degradation of physostigmine in human plasma is 76 nM (Hurst & Whelpton 1989) and a dissociation constant of 12·6 nM is not inconsistent with this. The hydrolysis of physostigmine by cholinesterase is complex, the rate of reaction being increased by the presence of substrate (Berry 1971). Watts & Wilkinson (1977) examined this in detail but it is not possible to deduce a value for the equilibrium constant for initial binding of physostigmine to the enzyme from their data. Using the data of Christenson (1969) the non-enzymic degradation of physostigmine at pH 7·4 and 20°C should be less than 1% over 20 h. The fact that in the presence of HSA the degradation was greater than this supports the hypothesis that there is a trace of a hydrolytic enzyme.

The other impurity that might be present in the Cohn Fraction V is α_1-acid glycoprotein. However, the fact that we were unable to demonstrate any binding to this macromolecule would appear to discount this as the binding site for physostigmine.

If our supposition is correct, then the binding of physostigmine to HSA is probably negligible. However, at therapeutic concentrations the binding to cholinesterase might have an important influence on the distribution of the drug.

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