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Correlation of Hydrophobicity with Protein Binding for Clorobiocin Analogs

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Abstract □ A new method of equilibrium dialysis was used to measure the binding of analogs of clorobiocin (18631 R.P.) to human serum albumin. Binding constants and numbers of binding sites on human serum albumin were calculated from the binding data and were used to calculate the percentage of compounds free in equilibrium with 4% albumin. Partition coefficients between *n*-octanol and phosphate buffer (0.05 *M*, pH 7.4) also were measured. A positive linear correlation ($r = 0.918$, $s = 0.240$, and $n = 10$) was obtained between log (bound/free compound) and log partition coefficient.

Keyphrases □ Hydrophobicity—clorobiocin analogs, correlation with protein binding □ Protein binding—clorobiocin analogs, correlation with hydrophobicity □ Clorobiocin analogs—correlation of hydrophobicity with protein binding

Clorobiocin (18631 R.P.) (1, 2) has been shown to be tightly bound to human serum albumin (3). In an effort to reduce this binding, various semisynthetic derivatives of the antibiotic were prepared, and the effect of these changes on the binding was monitored.

BACKGROUND

Binding of clorobiocin to human serum albumin was so high (99.97% with 10^{-5} *M* drug and 4% albumin) that the free drug concentration was in the range of 10^{-8}–10^{-9} *M*. To measure this drug level accurately by physicochemical means, radiolabeling of the drug would have been required. As an alternative, a method was developed whereby low concentrations of albumin were used so that the free drug concentration was sufficiently high for detection by UV spectrophotometry. From a plot of bound *versus* free drug, an iterative computer program gave binding constant values and the numbers of primary and secondary binding sites. These plots were used in a program that calculates the percentage of free drug in equilibrium with whole plasma.

The 4-hydroxycoumarin moiety probably contributes to the binding, since this group played a major part in the binding of the warfarin group of anticoagulants to human serum albumin (4). Various reports, listed by Jusko and Gretch (5), indicated that the hydrophobic nature was important for albumin binding. Accordingly, the partition coefficients of several clorobiocin derivatives were measured, and it was found that their hydrophobicity did play a major role in their binding to albumin.

EXPERIMENTAL

Equilibrium Dialysis—The technique was described initially by Coombs and Coulson (6). Two perspex blocks were screwed tightly together with a cellulose membrane[1] in between to form a complete unit with six compartments. Five units were used to hold 14 concentrations of compounds and two blanks (to allow for nonspecific UV absorbance).

For binding measurements, 2 ml of albumin[2] solution (0.25–2 mg/ml) was placed on one side of the membrane. The concentration of albumin appropriate for each compound was selected on the basis of the estimated partition using the Hansch aromatic substituent constants (7). The compounds[3] were dissolved in methanol at a concentration of 10 m*M* and diluted 1 in 50 in phosphate buffer (0.05 *M*, pH 7.4) to give the maximum concentration used. Further dilutions were made in the phosphate buffer. On the other side of the membrane was placed 2 ml of the compound solution (15–200 μM).

A plastic cap was fitted on each compartment to prevent evaporation. The units were shaken in a water bath for 8 hr at 37°, followed by 8 hr without shaking at room temperature. Aliquots from each side of the membrane were taken for the measurement of concentration by UV absorbance at a wavelength where there was no net change in absorbance on binding.

To examine the effect of methanol on the binding, methanolic and aqueous solutions of the sodium salts of clorobiocin and novobiocin were diluted in the buffer and tested; there was no significant difference in binding. The remaining compounds were dissolved initially in methanol since they were insoluble in water at higher concentrations.

Difference Spectra—The binding of each compound to human serum albumin was followed by UV spectroscopy as described previously (3) to find the wavelength for the measurement of the concentration of each compound, *i.e.*, to find the wavelength at which there was no net change in absorbance. Different albumin samples gave different results when binding clorobiocin derivatives according to the amount of dimer present. Therefore, albumin monomer was prepared using Sephadex G-100 as described previously (3).

Calculations—Binding constants and the number of binding sites were obtained from the binding data with an iterative computer program devised in these laboratories. The raw data were fitted to:

$$y = \frac{n_1 k_1 T}{1 + k_1 T} + \frac{n_2 k_2 T}{1 + k_2 T} \qquad \text{(Eq. 1)}$$

where y is the bound drug; T is the free drug; n_1 and n_2 are the numbers of primary and secondary binding sites, respectively; and k_1 and k_2 are the respective binding constants. The inclusion of a third set of binding sites did not improve the fit.

Arbitrary values for n_1, n_2, k_1, and k_2 were entered, and a standard deviation of the curve from the points was calculated. The value of n_1 is increased by 5%, k_1 and k_2 are kept constant, and the best value for n_2 is selected on the basis of a minimal standard deviation. If this standard deviation is lower than the previous one, then n_1 is increased further by

[1] Spectropor 2, M.S.E./Fisons, Crawley, Sussex, England.

[2] Miles.

[3] Clorobiocin was obtained from Dr. Jolles of Rhone Poulenc, novobiocin sodium was obtained from British Drug Houses, and the analogs were obtained from Dr. D. E. Wright, Dr. C. Smith, Dr. R. J. A. Walsh, and Mr. P. J. Warren of May and Baker Ltd. chemical research laboratories.

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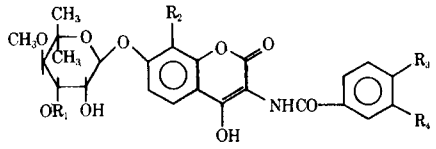
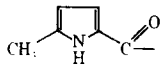
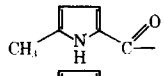
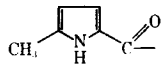
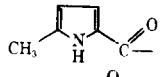
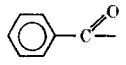
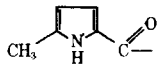
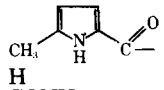


Table I—Binding and Partition Data for Clorobiocin Analogs and Human Serum Albumin

Compound	R_1	R_2	R_3	R_4	Number of Determinations	Average Minimal Standard Deviation of Iterations	Percent of Free[a] Compound	Bound/Free	Partition Coefficient
I		Cl	OCH_3	$CH_2CH{=}C(CH_3)_2$	3	1.21	0.01872	5340	776
II		Cl	OH	$CH_2CH_2CH(CH_3)_2$	4	2.25	0.03076	3250	617
Clorobiocin		Cl	OH	$CH_2CH{=}C(CH_3)_2$	4	1.33	0.02783	3592	162
Clorobiocin sodium		Cl	OH	$CH_2CH{=}C(CH_3)_2$	4	1.90	0.0568	1758	60.3
III		CH_3	OH	$CH_2CH{=}C(CH_3)_2$	2	0.99	0.0841	1188	115.0
IV		Cl	OCH_2-CH_2OH	$(CH_2)_2CH(CH_3)_2$	3	2.09	0.1079	926	229
V	H	Cl	OH	$CH_2CH{=}C(CH_3)_2$	2	1.49	0.216	462	7.24
VI		H	OH	$CH_2CH{=}C(CH_3)_2$	5	1.45	0.35	285	14.4
VII	H	CH_3	OH	$CH_2CH{=}(CH_3)_2$	2	0.99	0.64	155	6.03
Novobiocin	$CONH_2$	CH_3	OH	$CH_2CH{=}(CH_2)_2$	2	1.74	0.702	141.5	5.25

[a] Percent of free compound calculated at 40 mg/ml of human serum albumin and 10^{-5} *M* compound.

5%, and the best value for n_2 is obtained. If the second standard deviation is higher, then a 5% reduction in n_1 is made, and the calculation is carried out as before.

The values selected for n_1 continue to fall by 5% until the standard deviation starts to rise, at which point 1% increments are made to n_1 until the lowest standard deviation and corresponding n_2 value are obtained. This value for n_1 is retained while first k_1 and then k_2 are treated in the same way to achieve one complete iteration. Then the process is repeated. At the next stage, n_2 takes the place of n_1, and the process is repeated. A second iteration follows. The process is considered complete when an entire cycle of four iterations (two on n_1 and two on n_2) does not improve the standard deviation by more than 0.1%.

The minimum standard deviation obtained is reported in Table I as the average of two or more runs on each compound. The percentage of free compound at 10 μg/ml in equilibrium with 4% albumin then was calculated (8). This value indicated how much free compound would be present in plasma. The log of bound to free compound was plotted against the log partition coefficient by least-squares regression analysis. All programs were computed on a minicomputer[4].

Partition Coefficients—Partition coefficients between *n*-octanol and phosphate buffer (0.05 *M*, pH 7.4) were measured at five concentrations of the compound between 0.1 and 2.0 m*M*. The UV spectrum of the compound indicated the concentration in both octanol and the buffer. A standard curve was performed in both solvents. This procedure was adopted to detect any glass binding of the compounds, but none was found. The results were averaged (Table I).

Materials—The *n*-octanol was obtained commercially[5], and its absorbance was checked before use to show that it was less than one-tenth of a unit in the range of 270–315 nm[6]. Cellulose membranes were used in the equilibrium dialysis units[7] (Fig. 1).

RESULTS AND DISCUSSION

The partition data and the percentage of free compound in equilibrium with 4% albumin are listed in Table I. The partitions ranged from 1 to 776, and the percentage of free compound ranged from 0.02 to 0.71.

Although at neutral pH the clorobiocin series carried one negative charge due to the 4-hydroxycoumarin residue, the partition coefficients still were noticeably hydrophobic. This result may partially explain the tight binding of the series since, in general, albumin binding is related to hydrophobicity in a positive linear fashion as was shown for numerous series that bind to bovine and human serum albumin (5). These data are in agreement with those of O'Reilly (9), who found that the decrease in lipophilicity produced by the insertion of a hydroxy group into the 6, 7, or 8 position of the 4-hydroxycoumarin nucleus in warfarin reduced binding to human serum albumin of an order of magnitude (albeit at pH 10) when compared with warfarin. Clorobiocin and warfarin probably bind at the same site. In addition, human serum albumin is adapted to carrying anions of fatty acids and tryptophan among others. The methylpyrrole residue present in some derivatives clearly plays a major part in the binding, as is expected from such a hydrophobic structure.

The correlation coefficient for the graph of log bound to free compound against log partition coefficient is 0.918 (s = 0.240, n = 10, F = 43.0, and p = 0.0002), and the relationship is described by log (bound/free compound) = 0.633 log P + 1.839.

Bird and Marshall (10) used the ratio of bound to free compound in analyzing the binding data for 79 penicillin derivatives and human serum on the basis that the ratio was more meaningful and formally analogous to a partition coefficient. In addition, this ratio gave a more complete estimate of the binding, since calculation based on either the binding constant or the number of binding sites failed to indicate the extent of binding. Both parameters were required along with the data from secondary binding sites. The latter contribution was considerable in some cases. Dividing a small figure into a large one clearly will increase the small error in the percentage of free compound. To offset this error, a large number of points was taken to increase the accuracy of each determination.

The binding specificity is supported further by the positive intercept obtained. When the compound is distributed evenly between octanol and buffer (log P = 0), then more compound is bound to the albumin than to the buffer phase. This result implies either that albumin binding is much more hydrophobic than octanol or that some specific binding of these compounds is taking place (the partition then may be seen as an additional factor that modulates this binding). Support for the latter view may be taken from the intercepts obtained by Bird and Marshall (10),

[4] Wang system 2200 B.
[5] Specially pure grade, British Drug Houses.
[6] Spectrophotometric measurements were done on a Pye Unicam SP 800.
[7] Universal Scientific, London, England.

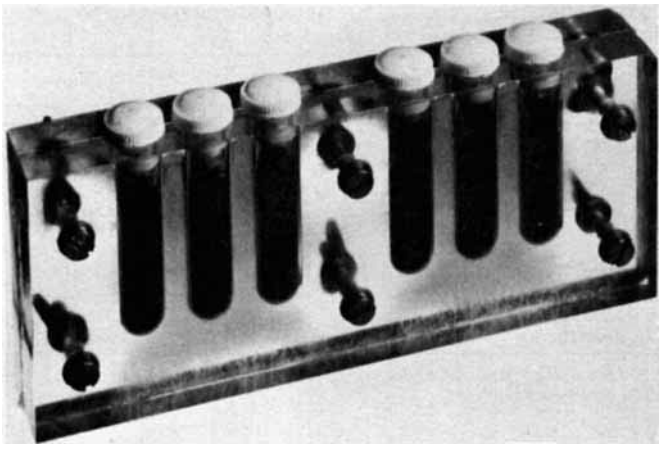


Figure 1—*Equilibrium dialysis units. (Reprinted with permission of the editors of Biochemical Society Transactions).*

Krieglstein *et al.* (11), who investigated the binding of 10 phenothiazines to bovine serum albumin, and Deutsch and Hansch (12), who studied barbiturate binding to the same protein. In each case, log (bound/free) was plotted; negative intercepts of 0.628, 0.578, and 1.22 were obtained, respectively. Penicillins, phenothiazines, and barbiturates apparently do not have as marked a binding site on albumin as do clorobiocins.

The difference in log P of 1.06 between VI and clorobiocin, which differ only by a chlorine atom, is only partially explained by the π value for aromatic chlorine of 0.71 (7), whereas the hydrophobic fragment constant of 0.92 (13) approaches it much more closely. Nevertheless, it seemed important to develop a mathematical relationship based on experimental results rather than on theoretical data for those complex molecules having a great deal of conformational interaction.

Salicylate binding to whole plasma gave a value of −1.32 for log P and 94% drug bound at a concentration of 13.8 μg/ml (6), a result that clearly does not fit the correlation obtained in this work. Other compounds with structures unrelated to either salicylate or clorobiocin also did not fit the correlation, which suggests that they might be binding at a different protein site. Sudlow *et al.* (14) reported that more than one binding site exists on human serum albumin for foreign compounds. The correlation obtained in this study applies to the clorobiocin derivative series. It also may apply to any structure that binds at the same site.

Use of the equilibrium dialysis units has facilitated accurate and reproducible work, which is difficult to carry out by dialysis in a large number of tubing bags. The alternative is to purchase an expensive instrument. The units described here combine the accuracy of the instrument with little more than the cost of the dialysis tubing. Used with the two computer programs, they estimate the binding to plasma albumin of drugs that are tightly bound and are not available in radioactive form. In addition, these units can be used to measure the binding of radioactive drug to whole plasma (6) and, potentially, the binding of any ligand to a macromolecule.

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Pharmacokinetics of Subcutaneous and Intramuscular Butorphanol in Dogs

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Abstract □ Butorphanol tartrate was administered intramuscularly and subcutaneously to adult male and female dogs at a dose of 0.25 mg/kg. No significant absorption lag time and no significant difference between peak intramuscular and subcutaneous serum concentrations were observed. The mean peak serum concentration was 29 ng/ml at mean times of 28 min after subcutaneous administration and 40 min after intramuscular administration. There were no significant differences in the pharmacokinetics of butorphanol in dogs with either route. The serum half-life was 1.62 hr, and the serum clearance was 3.45 liters/kg/hr. The apparent volume of distribution of butorphanol was 7.96 liters/kg. Although considerable inter- and intraanimal variation in C_{max} and AUC was observed, there was no significant difference in the area under the serum concentration *versus* time curves, and the two administration routes were considered bioequivalent.

Keyphrases □ Butorphanol—pharmacokinetics following subcutaneous and intramuscular administration, dogs □ Pharmacokinetics—butorphanol, subcutaneous and intramuscular administration, dogs □ Analgesics—butorphanol, pharmacokinetics following subcutaneous and intramuscular administration, dogs

Butorphanol tartrate [17-(cyclobutylmethyl)morphinan-3,14-diol tartrate], a new narcotic agonist–antagonist analgesic, is available[1] for use in humans and dogs (1). Previous studies indicated that loss processes account for a decrease in the extent of drug availability from extravascular parenteral injection sites (2). Deposition (3) and degradation (4) of the drug at the injection site can decrease both the rate and extent of absorption. The injection

[1] Stadol, Bristol Laboratories, Syracuse, N.Y.

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