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Binding of *N*-phenylanthranilic acid derivatives to bovine serum albumin

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The binding of drugs to extracellular protein, in particular, the albumin fraction of blood protein, has long been recognized as an important factor for consideration in drug design. Binding to serum albumin is known to affect the availability and level of response of certain pharmaceuticals. For example, phenylbutazone will displace warfarin bound to serum albumin in vivo and drastically affect its blood level and its anticoagulant effect (Solomon 1970). Other drugs are known to bind to specific sites on human albumin (Whitehouse et al 1971).

The binding of drugs to specific sites is the result of a number of possible types of interaction, e.g. electrostatic, hydrogen bonding and hydrophobic interaction. The objectives of physicochemical studies of drug/albumin binding is to isolate and quantitate the various contributions to binding that result from these interactions. An appropriate operational model for quantitating protein binding of drugs in terms of their physicochemical properties is the extra-thermodynamic model of Hansch & Fujita (1964).

The non steroidal antiinflammatory drugs, flufenamic acid [*N*-(α,α,α,-trifluoro-*m*-tolyl)anthranilic acid], meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl) anthranilic acid] and mefenamic acid [*N*-(2,3-xylyl) anthranilic acid] have been shown to bind to human serum albumin using a circular dichroic technique (Chignell 1969). The results suggested that both electrostatic and hydrophobic factors were involved in the binding of these drugs to human serum albumin. Attempts were made to determine the role of hydrophobic interactions in the binding of other *N*-phenyl-anthranilic acid derivatives to human serum albumin. This was done by looking at the correlation between the hexane–water partition coefficient of the *N*-phenylanthranilic acid and its association constant for binding to human serum albumin. Although some correlation was observed many compounds gave anomolous results. In this paper are reported the results of examining a wide range of substituted *N*-phenylanthranilic acids in an attempt to quantify the factors involved in the binding of the compounds to serum albumin.

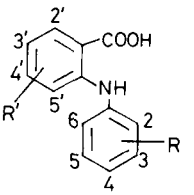
Materials and methods

Bovine serum albumin, globulin free, was purchased from the Sigma Chemical Company. Flufenamic acid, mechlofenamic acid and mefenamic acid were generously donated by Parke, Davis and Company.

All other substituted *N*-phenylanthranilic acids (Table 1) were synthesized by a modified Ullman synthesis (Ullman & Kipper 1907). Classical Ullman condensation of the appropriately substituted aniline with *o*-chlorobenzoic acid in the presence of a copper catalyst using iso-amyl alcohol as solvent gave consistently low yields (10%), however the following modification gave between 70–80% yields of substantially pure *N*-phenyl anthranilic acid.

o-Chlorobenzoic acid (0·134 M) was refluxed with the appropriately substituted aniline (0·196 M) in the presence of anhydrous potassium carbonate (0·181 M) and copper powder (100 mg) using dry dimethylformamide (100 ml) as solvent. Refluxing was carried out for 2 h, the catalyst filtered off and the filtrate acidified by the addition of dilute hydrochloric acid. After cooling the precipitated *N*-phenylanthranilic acid was

Table 1. Binding constants for the binding of substituted *N*-phenyl anthranilic acids to bovine serum albumin.



	Compound	$k^* \times 10^{-6}$	n^*
1	*N*-Phenyl anthranic acid	4·13	0·9
2	R = 4Cl	5·00	0·83
3	R = 4Br	5·12	0·87
4	R = $4OCH_3$	3·37	0·83
5	R = $4CH_3$	5·07	0·72
6	R = $4NO_2$	2·52	0·9
7	R = $4NH_2$	1·55	0·84
8	R = 3Cl	4·77	0·86
9	R = 3Br	5·67	0·83
10	R = $3CF_3$	5·40	0·86
11	R = $3OCH_3$	4·12	0·86
12	R = $3NO_2$	2·03	0·92
13	R = $3NH_2$	0·86	0·74
14	R = $2CH_3$	5·17	0·88
15	R = 2Cl	4·66	0·87
16	R = $2,3diCH_3$	5·50	0·72
17	R = 3,4diCl	3·94	0·90
18	R = 2,6diCl, $3CH_3$	3·41	0·82
19	R' = 4'Cl	4·40	0·90
20	R' = $4'NO_2$	2·34	0·96

* k = affinity constant for binding to bovine serum abumin.
n = number of binding sites/mole of bovine serum albumin estimated by Scatchard plot.

filtered off and recrystallized from methanol.

N-Phenyl-4-chloranthranilic acid (compound 19) and *N*-phenyl-4-nitroanthranilic acid) (compound 20) were synthesized in an analogous manner from 2,4-dichloro benzoic acid and 2-chloro-4-nitrobenzoic acid respectively. The recrystallized *N*-phenylanthranilic acids were identified by i.r., p.m.r. and mass spectroscopy and shown to be pure by t.l.c. in several solvent systems. The association constants for the binding of the *N*-phenylanthranilic acids to bovine serum albumin were measured by means of a spectrofluorimetric quenching technique using a Perkin Elmer MPF-3L fluorescence spectrophotometer (Table 1). A known concentration of bovine serum albumin (1 μm) in phosphate buffer (pH 7·4) was titrated against increasing concentrations of *N*-phenylanthranilic acid (0·00 μm–18·00 μm in dimethylformamide) until there was no further decrease in fluorescence of the bovine serum albumin (excitation wavelength 290 nm emission wavelength 344 nm). Dimethylformamide had no effect on the fluorescence of bovine serum albumin.

The concentrations of bound and unbound drug were calculated from the fluorescence quenching data (Chignell 1972) and the results evaluated using the Scatchard method (Scatchard 1949).

Results and discussion

The binding constants for 20 *N*-phenylanthranilic acids were estimated from the Scatchard plots of the spectrofluorimetric quenching data (Table 1). The compounds gave linear Scatchard plots with approximately one binding site mol^{-1} of protein. The binding constants were analysed by the Hansch linear free energy-related model using literature values for the physicochemical parameters (Norrington et al 1975), appropriate values being chosen to allow for variation in parameter value due to *ortho, meta* or *para* substitution.

Chignell (1969) showed, that with certain anomalies (notably with the 2,3-diCH_3 and the 2,6-diCl-3-CH_3 compounds) there was a linear relationship between the affinity constant for albumin binding and the n-hexane–water partition coefficient for a number of *N*-phenylanthranilic acids. Linear regression of certain of the compounds, including most of those investigated by Chignell, confirmed a linear relationship between the log of the binding affinity (log k) and the hydrophobic partitioning parameter (π) (eqn 1).

$$\log k = 0{\cdot}21\,\pi + 6{\cdot}53 \quad (\pm 0{\cdot}05) \qquad (1)$$

Compounds 1–5, 7–11, 13–15, 19, regression coefficient (R) = 0·947, variance test ratio (F) = 95, standard error (S) = 0·05.

However, certain compounds did not fit this simple linear relationship. These were the mononitro substituted compounds (6, 12 and 20) and the multiple substituted compounds (16, 17 and 18). Including these compounds in the simple linear regression equation results in equation 2.

$$\log k = 0{\cdot}199\,\pi + 6{\cdot}45 \quad (\pm 0{\cdot}085) \qquad (2)$$

Compounds 1–20, R = 0·76, F = 25, S =0.14.

This equation shows a very poor linear correlation between binding affinity and π. Obviously additional parameters must be employed to satisfactorily explain the binding of these additional compounds.

The multiple substituted compounds (16, 17 and 18) all had a much lower binding affinity for bovine serum albumin than might have been expected if the relationship between binding affinity and hydrophobicity was linear. This would suggest that these compounds either had exceeded an optimum hydrophobicity or that the additional substituents were having a steric effect hindering binding. These possibilities were investigated by adding either a parabolic (π^2) term to the equation (eqn 3) or by adding the steric parameter molar refraction (M.R.) to the equation (eqn 4). The MR parameter was chosen since it has been extensively tabulated, the values are not dependent on the position of substitution on the phenyl ring and there is no significant correlation between MR values and the values for the other parameters used in this work (π and δ) (Craig 1971).

$$\log k = -0{\cdot}13\,\pi^2 + 0{\cdot}24\,\pi + 6{\cdot}59 \quad (\pm 0{\cdot}1)\ (\pm 0{\cdot}12) \qquad (3)$$

compounds 1–5, 7–11, 13–19, R = 0·96, F = 88, S = 0·06.

$$\log k = -0{\cdot}04\,MR + 0{\cdot}27\,\pi + 6{\cdot}68 \quad (\pm 0{\cdot}18)\ (\pm(0{\cdot}13) \qquad (4)$$

compounds 1–5, 7–11, 13–19, R = 0·89, F = 25, S = 0·11.

It can be seen from these equations that inclusion of the parabolic π^2 term allows the multiple substituted compounds (16, 17 and 18) to be successfully predicted whilst inclusion of the steric molar refractivity term, MR, leads to no significant improvement in the correlation (compare eqns 2 and 4). Thus it can be concluded that the lower binding affinity of compounds 16, 17 and 18 is more likely to be due to a greater than optimum hydrophobicity than to an unfavourable steric effect.

The mononitro compounds (6, 12 and 20) were found to have a much lower binding affinity than would have been expected if there was a simple linear relationship between binding affinity and hydrophobicity since the nitro substituent has a reasonably high hydrophobicity (π for *para* NO_2 =0·22, π for *meta* NO_2 = 0·11). However the nitro group is a strong electron withdrawing group as characterized by the Hammett electronic parameter, σ (σ for para NO_2 = 1·24, σ for *meta* NO_2 = 0·71). Addition of σ to the simple linear equation allows the mononitro compounds to be successfully predicted (eqn 5).

$$\log k = 0{\cdot}32\,\pi - 0{\cdot}15\,\sigma + 6{\cdot}48 \quad (\pm 0{\cdot}09)\ (\pm 0{\cdot}05) \qquad (5)$$

Compounds 1–16, 19, 20, R = 0·94, F = 57, S = 0·07.

The combination of equations 3 and 5 leads to equation 6 which enables the binding affinity for bovine serum albumin of all 20 compounds to be modelled.

$$\log k = -0{\cdot}15\,\pi^2 + 0{\cdot}25\,\pi - 0{\cdot}19\,\sigma + 6{\cdot}55 \quad (6)$$
$$(\pm 0{\cdot}1) \quad (\pm 0{\cdot}12)\ (\pm 0{\cdot}07)$$

Compounds 1–20, R = 0·94, F = 62, S = 0·09.

To conclude, it has been shown that the binding of *N*-phenylanthranilic acids to bovine serum albumin is governed mainly by hydrophobic forces, probably by van der Waals' interactions between the phenyl ring and a hydrophobic area on the protein. The relationship is probably a parabolic one and addition of substituents with too great a hydrophobicity will lead to a decrease in binding affinity. Binding can also be modified by the presence of strong electron withdrawing substituents such as NO_2 which will reduce the availability of π electrons in the phenyl ring for van der Waals' interactions.

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Structural requirements for competitive α-adrenoceptor occupancy by cyclic and opened analogues of WB 4101

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Benzodioxanes represents one of the oldest class of reversible antagonists of the α-adrenoceptor (Melchiorre & Belleau 1981). Few of them were introduced in therapy but they were eventually discarded because of their severe adverse effects. All the compounds of this series have a benzodioxane nucleus as the main structural feature. Structure-activity relationships studies have shown that small structural manipulations may produce a pronounced difference in pharmacological activity. The most active of the series, WB 4101, was reported to be a very potent and selective postsynaptic α_1-blocker with an unusually high pA_2 value (Mottram & Kapur 1975; Kapur & Mottram 1978; Kapur et al 1978, 1979a, 1979b). It is being widely used for the characterization of the α_1-receptor through binding studies either in the brain or periphery (Kapur et al 1979b; Raisman et al 1979; Atlas & Adler 1981). Both benzodioxane and 2,6-dimethoxyphenoxyethyl moieties were reported to be essential for activity (Kapur et al 1978). In fact, the substitution of oxygen at position 4 with a methylene gives rise to a significant decrease in activity. On the other hand, changing the ethoxy moiety, or removal of one or both methoxy groups, greatly reduces the potency compared with WB 4101. One aspect of structure-activity relationships which appear to have escaped attention until now concerns the possibility that the target site of WB 4101 may have multiple identical or quasi-indentical subsites.

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In fact, a close inspection of the chemical structure of WB 4101 reveals that the benzodioxane nucleus may be regarded as the desmethoxy cyclic moiety of the 2,6-dimethoxyphenoxymethyl group and vice versa. Thus a symmetric or quasi-symmetric molecule can be designed which might promote a better fit with the adrenergic α-receptor. Dibozane, another α-antagonist of benzodioxanes class, is actually a symmetric molecule. However, it incorporates two basic nitrogens and hence the distance between the two benzodioxane nuclei is longer than that of WB 4101 (Melchiorre & Belleau 1981). Nevertheless, dibozane displays high α-blocking activity which may suggest the interaction with symmetric sites. This reasoning may be justified if one considers that the α-receptor surface incorporates symmetrically at least four anionic sites linearly arranged near a buried target thiol (Melchiorre 1981; Melchiorre & Belleau 1981). Thus, it is theoretically possible that the receptor surface, in addition to these anionic sites, might well incorporate other (aromatic) sites disposed symmetrically. With this goal in mind, we have evaluated some drugs structurally related to WB 4101.

Method

Vasa deferentia from male albino rats (175–200 g) were mounted individually in 10 ml organ baths containing Krebs bicarbonate buffer. The medium was maintained at 37 °C while being aerated with 95% O_2 – 5% CO_2.