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INTERACTION OF DIFTALONE AND SOME OF ITS METABOLITES WITH HUMAN SERUM PROTEINS

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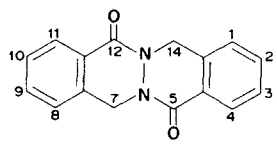
Abstract—The ability of a novel anti-inflammatory drug, diftalone* and some of its metabolites to interact with human serum proteins has been studied. By gel filtration, albumin has been identified as the protein fraction responsible for the binding. The association constants were determined *in vitro* by means of equilibrium dialysis. In addition, the temperature and pH dependence of the association constants have been investigated. These data were obtained as a preliminary evaluating binding influence *in vivo*.

In the present work, a new type of anti-inflammatory drug, diftalone (phthalazino [2,3-b]phthalazine-5,12 (7H, 14H-dione) [1], with the following structure: and two of its metabolites found in man, have been studied for the characteristics of their interaction with human serum proteins. The two metabolites considered were: the 7-hydroxy-derivative of diftalone, the main metabolite in human plasma (demonstrated also in many other animal species), and the 7-hydroxy-glucuronide, the main urinary metabolite for all the species so far investigated and found also in moderate amounts in plasma of dogs (data to be published).



The purpose of the work was (1) to verify the interaction between diftalone and its metabolites with the human serum proteins, (2) to identify the protein fraction involved in these interaction, and (3) to determine some of the quantitative parameters of the binding such as the association constants, and the number of the interacting binding sites.

MATERIALS AND METHODS

Chemicals. [5,12-^{14}C]. Diftalone, sp. act. 3·57 mCi/m-mole was kindly supplied by Dr. R. White, Lepetit Milano. The [5,12-^{14}C]7-hydroxy-derivative, sp. act. 0·118 mCi/m-mole, and [5,12-^{14}C]7-hydroxy-glucuronide, sp. act. 0·052 mCi/m-mole, were biosynthetically prepared by the authors.

Human serum albumin (HSA), 98% electrophoretically pure, was purchased from Sigma Chemical Co. (Cohn's fraction V). All calculations are reported in terms of a molecular weight of 69,000 for HSA.

Pipes buffer, piperazine *N-N'*-bis (2-ethane sulfonic acid) sodium salt·monohydrate, A grade, Calbiochem. Tris buffer, hydroxy-methyl-aminomethane, reagent grade, Sigma Chemical Co.

Radioactivity measurements. A Packard model Tri-Carb 3330 liquid-scintillation spectrometer was employed for the radioactivity measurements, and the scintillation cocktail Instagel was used.

Column chromatography experiments. Gel filtration of serum proteins on Sephadex G-200, was performed according to the method of Flodin and Killander [2]. The relevant chromatographic conditions were: volume of the moving phase V_0 = 75 ml; volume of the stationary phase V_t = 260 ml; eluent, 0·1 M Tris–HCl containing 0·1 M NaCl, pH 8·1; flow rate 0·32 ml/min; temperature 22°.

Ion exchange chromatography on DEAE Sephadex A-50 [3] was used to separate albumin from transferrin, elution being accomplished at pH 8·0 with Tris–HCl buffer using a linear gradient from 0·05 to 0·5 M. The purity of fractions was checked by immunological assay with anti-human transferrin (Partigen M Behringwerke).

Equilibrium dialysis. For these experiments, a multiple-equilibrium dialysis system (Dianorm, Innovativ Medizine, Esslingen, Zurich) was employed [4]. The operational conditions for the macro-dialysing cells were: total volume of half cell 1·36 ml; dialysing volume (V) 1·00 ml; membrane surface area (A) 4·52 cm^2; Q factor A/V 4·5. A gentle rotation of the dialysis cells (15 rev/min) was used to avoid any possible denaturation of the biological material and to reach the equilibrium more rapidly. The dialysis experiments were performed between 0° and 37°. As dialysing membranes, open Visking tubings, thickness 0·025 mm, were used after appropriate pretreatment (washed 15 min in distilled water, 20 min in 30% ethanol, rinsed again for 30 min in distilled water, and finally washed twice in the Pipes buffer solution 0·02 M, pH 7·4). Diftalone and 7-hydroxy-diftalone were added to the dialysing medium dissolved in 20 μl of ethyl alcohol, because of their low solubility in water.

* Aldione®, Lepetit S.p.A.

RESULTS

Identification of the protein fraction which interacts with diftalone and its metabolites. In a preliminary investigation, plasma from subjects treated with a single oral dose (7 mg/kg) of [^{14}C]diftalone, collected 6, 10 and 24 hr after the administration, were chromatographed on Sephadex G-200 to identify the protein fraction binding the unchanged drug and metabolites. At these three times, two metabolites besides diftalone were found in human plasma, 7-hydroxy-diftalone and a 7-14-hydroxy-derivative of diftalone; no evidence is yet available for the presence of the 7-hydroxy-glucuronide in the human (data to be published).

The gel-filtration experiments with the different plasmas always indicated that under the experimental conditions used the albumin–transferrin fraction was the only one interacting with diftalone and its metabolites, (Fig. 1). In order to determine whether albumin or transferrin was the protein fraction involved in the interaction with diftalone and its metabolites, the albumin–transferrin fraction eluted from the G-200 column was chromatographed on DEAE-Sephadex A-50. The analysis of the eluate (Fig. 2) shows one radioactive peak only, associated with the albumin fraction.

Calculation of the association constants of diftalone, 7-hydroxy-diftalone and 7-hydroxy-glucuronide with human albumin. The association constants have been calculated from equilibrium dialysis data. Using a constant pH of 7·4, and a temperature of 37°, the other working conditions were selected after some initial experimentation. For example, Pipes was found to give higher binding than other buffers (e.g. phosphate) and 0·02 M buffer was chosen because high molarity (up to 0·1 M) inhibits binding. Using these conditions, dialysis times for equilibrium were experimentally determined for each ligand at the different temperatures considered, with and without proteins. The actual association constants were graphically derived from the Scatchard plots (bound/free versus bound) according to the equation [5]:

$$[b]/[f] = K_a(n[M] - [b])$$

where M = protein molarity, $[b]$ = bound, $[f]$ = free, K_a = association constant, n = maximum number of ligand molecules that can be bound per protein molecule.

The Scatchard plots of the interactions between albumin and diftalone, and albumin and the 7-dihydroxy compound using 0·02 M Pipes, pH 7·4, are shown in Fig. 3 and 4, respectively. The binding parameters derived: these plots are: diftalone albumin, n = 1·38, $K_{a_1} = 3{\cdot}85 \times 10^4$ liters mole^{-1}, $K_{a_1} \cdot n = 5{\cdot}31 \times 10^4$ liters mole^{-1}; 7-hydroxy diftalone albumin, n = 1·42, $K_{a_1} = 0{\cdot}62 \times 10^4$ liters mole^{-1}, $K_{a_1} \cdot n = 0{\cdot}88 \times 10^4$ liters mole^{-1}.

The graphs were fitted by the method of the least squares. Due to the low solubility of the two compounds, and to their relatively low specific activities, it was impossible to investigate a broader range of concentrations: Thus, the association constants calculated must be attributed to the 'strong site' only. However, for both diftalone and 7-hydroxy-diftalone, the existence of more than one binding site per albumin molecule cannot be excluded.

Attempts to measure the association constant of the 7-hydroxy-glucuronide by equilibrium dialysis at 37° were unsatisfactory because of the low affinity of this conjugate even for albumin.

Nature of the binding. The formation of the protein–ligand complex results in a change in free energy ΔF, which is exponentially related to the association constant by the equation:

$$\Delta F = RT - \ln K_a$$

In evaluating the forces involved in the binding of a ligand by protein, it is often useful to determine whether the free energy change comes about from a change in heat content (ΔH) or in the entropy of the system (ΔS). Assuming ΔH being constant over the temperature range studied:

$$\frac{d(\ln K_a)}{dT} = \frac{\Delta H}{RT^2}, \text{ or in the integrated form:}$$

$$\ln K_a = -\frac{\Delta H}{RT} + C.$$

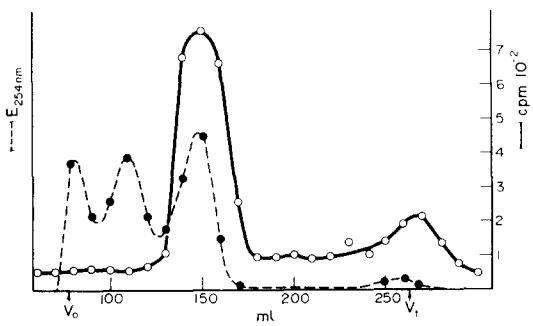


Fig. 1. Gel filtration on Sephadex G-200 of a 2 ml serum sample taken 8 hr after the oral administration of 500 mg [^{14}C]diftalone (100 μCi). Eluent 0·1 M Tris–HCl, pH 8·1 containing 0·1 M NaCl, flow rate 0·32 ml/min, temperature 22°. Dotted line, extinction at 254 mm; solid line, total radioactivity of each fraction.

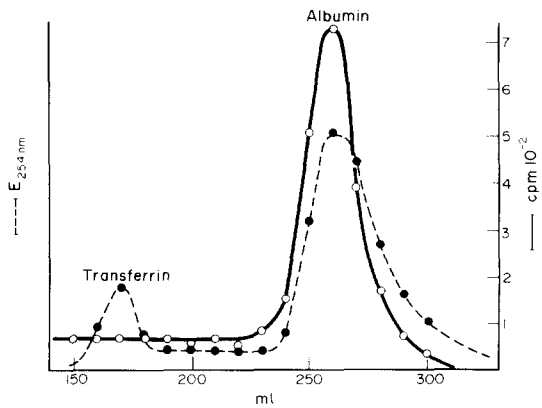


Fig. 2. Chromatography on DEAE-Sephadex A-50 of the 'albumin transferrin' fraction previously separated on a G-200 column. Eluent Tris HCl, pH 8·0, with a linear gradient from 0·5 M to 0·5 M; flow rate 0·5 ml/min. Dotted line, extinction recorded at 254 nm; solid line, total radioactivity of each fraction.

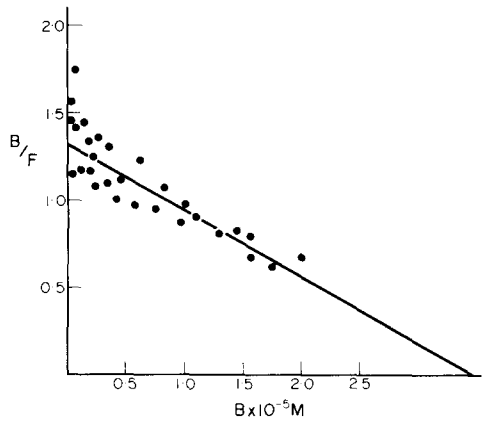


Fig. 3. Scatchard plot of the binding of diftalone at 37°, pH 7·4, with HSA. Albumin 2·5 × 10^{-5} M. Dialysis time 8 hr.

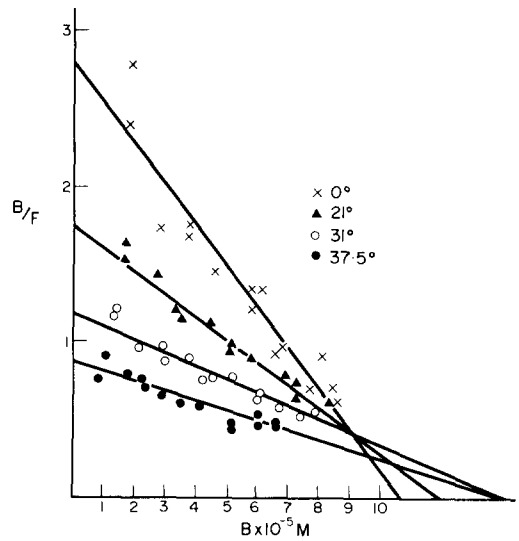


Fig. 4. Scatchard plots at different temperatures of the binding of 7-hydroxy-diftalone at pH 7·4, with HSA. Albumin 10^{-4} M. Dialysis time 6 hr.

from the plot of ln K_a vs $1/T$, the slope $-\Delta H/R$ can be calculated. Finally, the entropy change is given by the equation:

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ$$

As listed in Table 1, the association constants of diftalone and 7-hydroxy diftalone with HSA, calculated at different temperatures (0·02 M Pipes, pH 7·4), gave values at 0° of about ten times those obtained at 37°. Moreover, for both the compounds, the plots of ln K_a vs $1/T$ were linear within the range of temperatures between 22° and 37° (Fig. 5). The values of ΔF°, ΔH° and ΔS° calculated within this temperature interval are summarized in Table 2.

At 0°, the association constants of the two compounds under investigation, deviated from the verified linear relationship ln K_a vs $1/T$. These deviations, however, might be explained either by a conformational change of the macromolecules, or, more probable at this temperature, by a modification of the solvation volumes of groups involved in the binding process.

Although the variation of n, as determined experimentally, makes the estimation of the therodynamic parameters only approximate, the thermodynamic interpretation remains correct. These results tend to indicate that both the compounds interact with HSA by polar binding, in support of this hypothesis, the interaction of diftalone with HSA was demonstrated to be strongly pH-dependent (Fig. 6).

DISCUSSION

Our results can be summarized as follows:

(a) Diftalone and its metabolites interact with human serum proteins, and this interaction is mainly with the albumin fraction.

(b) Diftalone shows a higher binding force than the two metabolites considered, the 7-hydroxy-glucuronide particularly is bound very weakly.

(c) The association constant for diftalone, 3·85 × 10^4, (37°, pH 7·4) under the optimal experimental conditions used, is relatively weak, as has been found for many other common anti-inflammatory drugs,

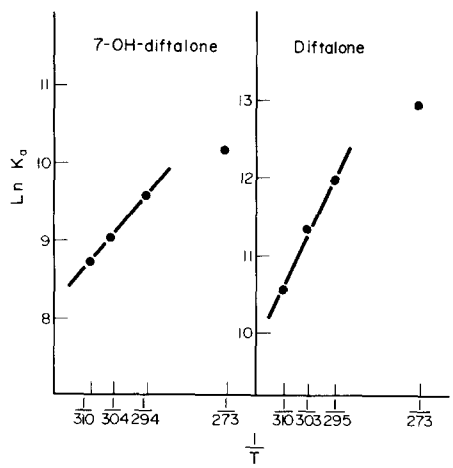


Fig. 5. In K_a as a function of $1/T$ for diftalone and 7-OH-diftalone HSA complexes; pH 7·4; HSA 2·5 × 10^{-5} M.

Table 1. Influence of temperature on the association constants of diftalone and 7-OH-diftalone with HSA

Diftalone			7-OH-diftalone		
T (°)	n	K_a (liters mole^{-1})	T (°)	n	K_a (liters mole^{-1})
37	1·38	3·85 × 10^4	37	1·42	0·62 × 10^4
30	1·12	8·35 × 10^4	31	1·40	0·84 × 10^4
22	0·98	1·52 ± 10^5	21	1·20	1·45 × 10^4
0	0·76	4·03 × 10^5	0	1·06	2·58 × 10^4

Table 2. Changes in free energy, ΔF, enthalpy, ΔH, and entropy ΔS of the diftalone HSA and 7-OH diftalone HSA complexes

	Diftalone	7-OH-diftalone
ΔF (cal/mole ligand bound)	−6504	−5379
ΔH (cal/mole ligand bound)	−15843	−9647
ΔS (cal/mole degree)	−30·13	−13·77

(phenylbutazone [6], 22°, pH 7·4, $K_{a_1} = 1{\cdot}17 \times 10^5$; indomethacin [7], 37°, pH 7·4, $K_{a_1} = 1{\cdot}32 \times 10^4$; aspirin [8], 25°, pH 7·0, $K_a = 2 \times 10^5$).

(d) The column chromatography experiments, (Figs. 1 and 2), in spite of the weak association constants determined for diftalone and 7-hydroxy-diftalone did not show large dissociation phenomena, and therefore probably indicate that dissociation rates are slow for the ligand–protein complexes.

(e) The interaction between diftalone and 7-hydroxy-diftalone with human albumin are strongly temperature-dependent. Therefore it can be concluded that Van der Walls or hydrogen-bonding forces are probably involved. However, further studies with other techniques (e.g., microcalorimetry) would have to be carried out to determine whether conformational changes of albumin are also involved in the binding process.

In conclusion, because of the low association constant value, of diftalone and 7-OH-diftalone with HSA, the authors believe the influence of protein binding of these compounds on their pharmacokinetics should not be very important *in vivo*, but knowledge of the distribution volumes will be important to determine this. Further studies are in progress to investigate more extensively the protein binding of diftalone, either in serum or in tissues, trying to correlate its properties with those of other non-steroidal anti-inflammatory drugs.

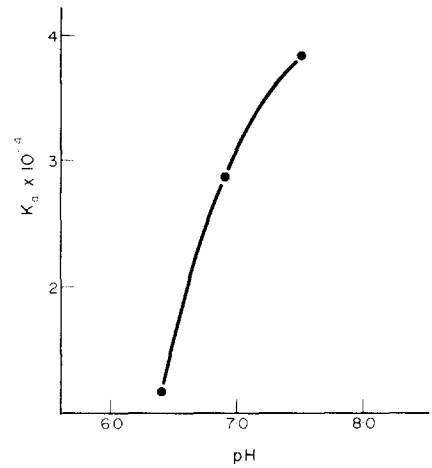


Fig. 6. pH dependence of diftalone–HSA association constant, at 37°; HSA $2{\cdot}5 \times 10^{-5}$ M.

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