International Journal of Pharmaceutics, 42 (1988) 225–234
Elsevier

IJP 01432

Comparative study of binding of an antiallergic substance to serum albumin and erythrocytes in man, in the dog and in the rat

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(Received 7 May 1987)
(Accepted 1 September 1987)

Key words: Erythrocyte; Serum albumin; Drug binding parameter

Summary

The binding to red blood cells of a synthetic compound (F 1865; ethyl-3-methoxyphenyl-4-thiazolyl-2-oxamate) only very slightly soluble in water was studied in man, dog and rat. Binding of the same compound to serum albumin was studied in man and in the dog. Distribution of the compound between serum albumin and erythrocytes at physiological concentrations was studied in man and in the dog. A simple model integrating the two steady-state bindings was used to link these measurements of distribution to physicochemical binding parameters. This method confirmed values for parameters of binding to serum albumin obtained with great difficulty of equilibrium dialysis, bearing in mind the poor solubility of the compound and the binding to the dialysis membrane.

Introduction

It is known that a given concentration of a drug in whole blood is associated with a concentration of the circulating free form which depends essentially on the distribution of the compound between plasma proteins and blood cells (Ehrnebo, 1986; Garrett and Lambert, 1973; Perrin, 1986; Wallace and Riegelman, 1977). The importance of binding studies is today unanimously recognized (Fromson , 1986; Routledge, 1986; Sjöqvist, 1986). In many cases binding to serum albumin alone serves to indicate the behaviour of the compound, binding to other blood consituents being minimal (Meyer and Guttman, 1986; Tillement et al., 1984). However, in the case of certain hydrophobic compounds which are only minimally soluble in water, binding studies become more complex since in such compounds, that are somewhat rejected from the aqueous medium, binding occurs not only to proteins but also to blood cells (Altmayer and Garrett, 1983; Derendorf and Garrett, 1983; Garrett and Hunt, 1974).

The present study involved a determination of the binding parameters of a very slightly hydrosoluble synthetic compound to red blood cells and serum albumin in man and in two animal species, the dog and the rat. It was not possible to study binding to serum albumin in the rat for technical reasons. These values were then used to compare theoretical and experimental distributions of the compound in the erythrocyte/serum albumin system at physiological concentrations (Fournier, 1986).

Materials and Methods

F 1865 (ethyl-3-methoxy-phenyl-4-thiazolyl-2-oxamate)

The compound studied was synthesized by Pierre Fabre Laboratories. It was given the code F

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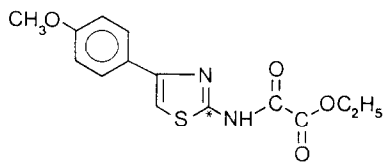

Scheme 1. The compound was labelled with ^{14}C between the N and S atoms.

1865. Its molecular weight is 306.3 and its structural formula is as follows:

This molecule belongs to the series of phenyl-4-thiazolyl-2-oxamates, which compounds exhibit antiallergic properties after oral administration (Tarayre et al., 1982).

The limit of solubility of the compound in water was of the order of 6 mg/liter, i.e. $2 \cdot 10^{-5}$ M.

Because of this low solubility and for reasons of accuracy in measurements of radioactivity, it was not possible to dilute the labelled compound with cold compound. The specific activity of the compound was $11{,}968 \cdot 10^{12}$ dpm/mol or $15{,}187 \cdot 10^{12}$dpm/mol according to manufacturing batches.

Once again, because of the low solubility of the compound, solutions of F 1865 were obtained from a stock solution previously filtered through a Millex GS 0.22-μm Millipore filter.

Chemical and radiochemical purity of the compound were confirmed periodically by thin-layer chromatography ethyl acetate/petroleum or butanol ether/acetic acid/water and mass spectrography.

Buffers

Measurements of binding to serum albumin were made in Sörensen phosphate buffer, pH 7.4, ionic strength 0.174.

Measurements of binding to red cells were carried out in isotonic phosphate buffer pH 7.4, ionic strength 0.255.

Human serum albumin (HSA)

A 1887 Sigma, fatty acid-free, $M_r = 66{,}241$.

Dog serum albumin (DSA)

A 9263 Sigma, $M_r = 66{,}000$.

Red blood cells

Human red blood cells (HRBC) of group 0^+ were supplied by the Montpellier Transfusion Center in the form of peak cells stored with ACD medium.

Beagle dog red blood cells (DRBC) were obtained from whole blood drawn into citrate tubes from the forepaw vein of the conscious animal.

CD-strain rat red blood cells (RRBC) were obtained from whole blood drawn into citrate tubes by intracardiac puncture in the animal anesthetized with ether.

Before each use, red blood cells were washed 2–3 times with buffer until the supernatant was clear and colorless, in order to obtain packed cells with a 100% erythrocyte concentration.

Equilibrium dialysis

The Dianorm (Diachema A.G., Zurich) automatic apparatus was used. Dialysis cells used were Macro 15, each with a volume of 1 ml and a membrane area of 11.3 cm^2. The two compartments were separated by a semipermeable membrane of cellulose (Diachema 10–15), the retention limit of which corresponds to molecular weights of 10,000 Da. Dialysis cells were mixed constantly at a low rate of 4 rpm in such a way as to decrease risks of hemolysis when working with erythrocytes. The entire Dianorm system was placed in a chamber heated at 38° C for 3 h (time determined by dialysis of the compound at different concentrations vs pure buffer).

The method of equilibrium dialysis was used above all to study binding to serum albumin and secondarily binding to red blood cells, but also to determine the free fraction in experiments involving distribution of the compound between serum albumin and erythrocytes at physiological concentrations.

Sedimentation

When studying binding of a ligand to blood cells, separation between bound compound and free compound may be obtained by simple sedimentation of cells. This thereby eliminates all problems related to parasitic binding, e.g. to the dialysis membrane.

After incubation of compounds at 37° C for 3 h with moderate mixing in stoppered centrifuge tubes, separation can be obtained by centrifugation at 3000 rpm for 10 min. The compound is then measured in the red blood cell pellet and the supernatant fluid.

Measurement of concentrations of ^{14}C-labelled compound by liquid scintillation

A Packard Tricarb 3255 spectrometer was used. Liquid samples (solutions of F 1865, serum albumin solution or supernatant of an erythrocyte phase) were counted as such in the presence of Instagel (1 ml of liquid per 10 ml of Instagel).

For red blood cell pellets, an aliquot specimen (about 10 mg) was digested in 1 ml of soluene/propanol (50/50), and decolored by addition of 0.5 ml of 110 vols. H_2O_2, then addition of 10 ml of Instagel/HCl.

Counting yields from red blood cell pellets were always poor in comparison to those obtained with supernatant fluids. Preference was given to calculation of concentrations of compound bound to red blood cells by the difference between the initial total concentration and concentration in the supernatant after centrifugation.

Method of calculation of binding parameters

Exploitation of experimental binding data was made according to the transformation of Scatchard (1949). Experimental data were treated numerically using an Apple IIE microcomputer. Linear isotherms were evaluated by simple linear regression whereas non-linear isotherms were treated using a model with two categories of sites by the method of Rosenthal (1967). The software used provided curves of saturation and of calculated isotherms as well as values of the binding parameters n and K.

Results

Binding of F 1865 to HSA (Fig. 1)

Binding of F 1865 to HSA at 40 g/liter. HSA concentration: 40 g/liter, i.e. $6.04 \cdot 10^{-4}$ M. F 1865 concentration: 0.3 to 2.7 mg/liter, i.e. $9 \cdot 10^{-7}$ to $9 \cdot 10^{-6}$ M.

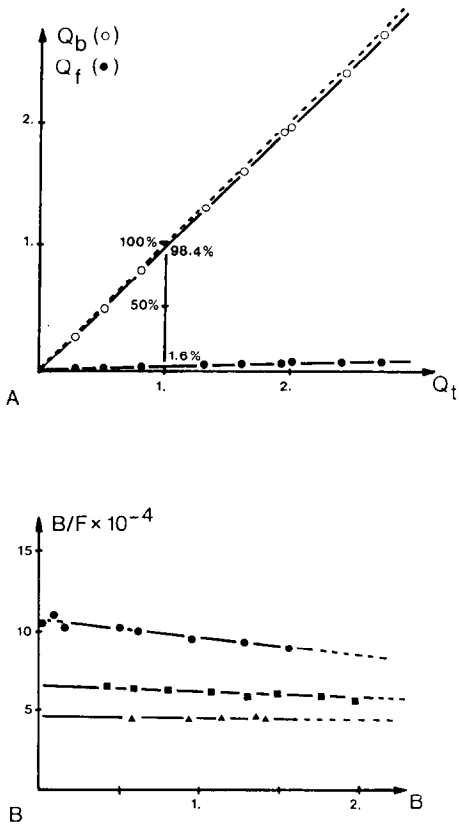

Fig. 1. Binding of F 1865 to HSA. A: binding of F 1865 to HSA 40 g/liter. *X*-axis: total concentration of F 1865 in 40 g/liter HSA solution: Q_t (mg/liter). *Y*-axis: concentration of F 1865 bound to HSA: Q_b (mg/liter); concentration of free F 1865: Q_f (mg/liter). B: Scatchard plots of the binding of F 1865 to HSA in the presence of ethanol at various concentrations: 2.5%, (●); 5%, (■); 10%, (▲). *B*, mol F 1865 bound per mol of HSA; *F*, free concentration of F 1865 (M).

Results: bound fraction 98.4%;
free fraction 1.6%.

Binding isotherm — Scatchard representation. HSA concentration: 0.8 g/liter, i.e. $1.21 \cdot 10^{-5}$ M. F 1865 concentrations: 0.2 mg/liter to 20 mg/liter, i.e. $6.53 \cdot 10^{-7}$ M to $6.53 \cdot 10^{-6}$ M. Because of problems related to the solubility of the compound, we worked with 3 concentrations of ethanol: 2.5%, 5% and 10%. Extrapolation to a nil concentration of ethanol gave the following value for binding parameters:

$n = 1$ mol/mol of HSA;

$K_a = 17.5 \cdot 10^4$ mol^{-1}.

Binding of F 1865 to DSA (Fig. 2)

Binding of F 1865 to DSA at 40 g/liter. DSA concentration: 40 g/liter, i.e. $6.06 \cdot 10^{-4}$ M. F 1865 concentrations: 0.15 mg/liter to 1.6 mg/liter, i.e. $4.9 \cdot 10^{-7}$ M to $5.2 \cdot 10^{-6}$ M.

Results: bound fraction 94%;
free fraction 6%.

Binding isotherm — Scatchard representation. DSA concentration: 40 g/liter, i.e. $6.06 \cdot 10^{-5}$ M. F 1865 concentrations: 0.2 to 2 mg/liter, i.e. $6.53 \cdot 10^{-7}$ M to $6.53 \cdot 10^{-6}$ M. In the case of DSA it was possible to obtain the binding isotherm directly, though we also worked with 10% and 15% ethanol in order to confirm adequate correspondence between direct values and extrapolated values. Values for binding parameters were as follows:

$n = 1.5$ mol/mol of DSA;

$K_a = 2.38 \cdot 10^4$ mol^{-1}.

Binding of F 1865 to HRBC (Fig. 3)

Binding of F 1865 to HRBC at a hematocrit of 45%. F 1865 concentrations: 0.015 to 2.2 mg/liter

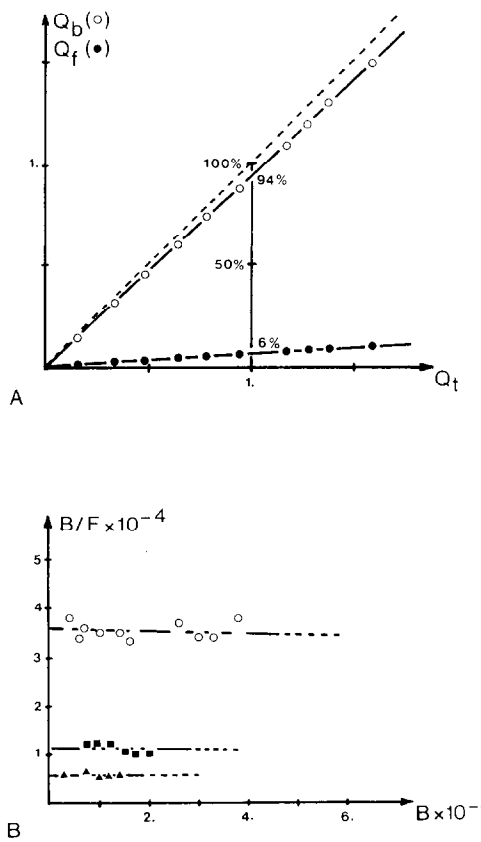

Fig. 2. Binding of F 1865 to DSA. A: binding of F 1865 to DSA at 40 g/liter. *X*-axis: total concentration of F 1865 in 40 g/liter DSA solution: Q_t (mg/liter). *Y*-axis: concentration of F 1865 bound to DSA: Q_b (mg/liter); concentration of free F 1865: Q_f (mg/liter). B: Scatchard plots of the binding of F 1865 to DSA alone (○) and in the presence of 10% (■) and 15% (▲) of absolute ethanol. *B*, mol of F 1865 bound per mol of DSA; *F*, free concentration of F 1865 (M).

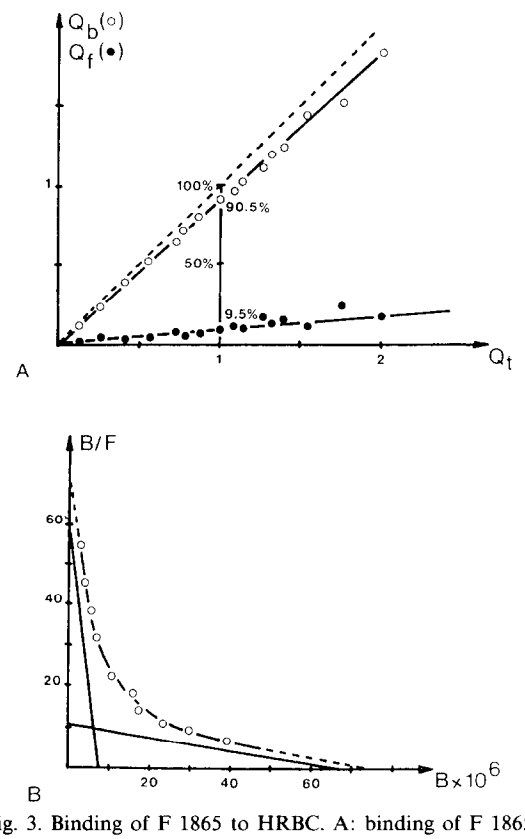

Fig. 3. Binding of F 1865 to HRBC. A: binding of F 1865 to HRBC (hematocrit 45%). *X*-axis: total concentration of F 1865 in HRBC suspension: Q_t (mg/liter). *Y*-axis: recovered quantity of F 1865 (mg) in 450 ml of packed red blood cells: Q_b; recovered quantity of F 1865 (mg) in 550 ml ml of buffer solution: Q_f. B: Scatchard plot of the binding of F 1865 to HRBC. *B*, mol of F 1865 bound per liter of packed red blood cells; *F*, free concentration of F 1865 (M red blood cell suspension).

of red blood cell suspension, i.e. $5.06 \cdot 10^{-8}$ to $7.23 \cdot 10^{-6}$ M.

Results: two methods were used.

Sedimentation:	bound fraction	90.5%,
	free fraction	9.5%.
Equilibrium dialysis:	bound fraction	93.5%;
	free fraction	6.8%.

A difference of the order of 3% occurred due to binding of F 1865 to the dialysis membrane.

Binding isotherm — Scatchard representation. Hematocrits: 4–12% (4–5–6–7–8–9–10–11–12%). F 1865 concentrations: 0.025–7.67 mg/liter of red blood cell suspension, i.e. $8.2 \cdot 10^{-8}$ to $2.49 \cdot 10^{-5}$ M.

Results: two categories of sites:

high-affinity binding sites with $n_1 = 7.57 \cdot 10^{-6}$ M of packed red blood cells and $K_1 = 7.69 \cdot 10^{6}$ mol^{-1};

low-affinity binding sites with $n_2 = 6.48 \cdot 10^{-6}$ M of packed red blood cells and $K_2 = 1.67 \cdot 10^{6}$ mol^{-1}.

Binding of F 1865 to DRBC (Fig. 4)

Binding of F 1865 to DRBC at a hematocrit of 45%. Concentrations of F 1865 varied from 0.095 to 1.2 mg/liter of red blood cell suspension, i.e. $3.1 \cdot 10^{-7}$ to $3.9 \cdot 10^{-6}$ M.

Results: bound fraction 91%;
free fraction 9%.

(N.B.: both methods, sedimentation and equilibrium dialysis, gave identical results which were certainly due to stronger binding to DRBC which shifted the binding of the compound to the dialysis membrane.)

Binding isotherm — Scatchard representation. Hematocrits: 5–45% (5–20–45%). F 1865 concentrations: 0.02 to 1.3 mg/liter of red blood cell suspension, i.e. $6.53 \cdot 10^{-8}$ to $4.24 \cdot 10^{-6}$ M.

Results: two categories of sites:

high-affinity binding sites with $n_1 = 0.986 \cdot 10^{-6}$ M of packed red blood cells and $K_1 = 21.9 \cdot 10^{6}$ mol^{-1};

low-affinity binding sites with $n_2 = 3.36 \cdot 10^{-5}$ M of packed red blood cells and $K_2 = 4.88 \cdot 10^{5}$ mol^{-1}.

Binding of F 1865 to RRBC (Fig. 5)

Binding of F 1865 to RRBC at a hematocrit of 45%. F 1865 concentrations: 0.1–0.87 mg/liter of red blood cell suspension, i.e. $3.2 \cdot 10^{-7}$ to $2.85 \cdot 10^{-6}$ M.

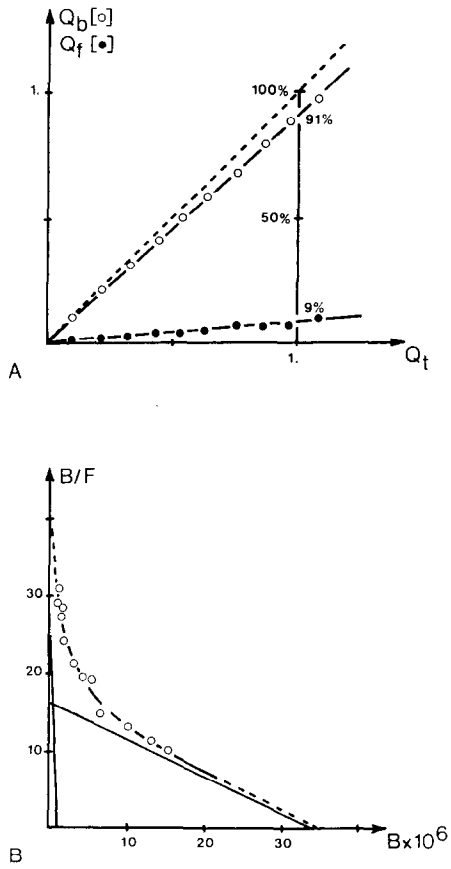

Fig. 4. Binding of F 1865 to DRBC. A: binding of F 1865 to DRBC (hematocrit 45%). *X*-axis: total concentration of F 1865 in DRBC suspension: Q_t (mg/liter); *Y*-axis; recovered quantity of F 1865 (mg) in 450 ml of packed red blood cells: Q_b; recovered quantity of F 1865 (mg) in 550 ml of buffer solution: Q_f. B: Scatchard plot of the binding of F 1865 to DRBC. *B*, mol of F 1865 bound per liter of packed red blood cells; *F*, free concentration of F 1865 (M red blood cell suspension).

Results: sedimentation method:
bound fraction 90.5%;
free fraction 9.5%.

Binding isotherm — Scatchard representation. Hematocrits: 5–45% (5–25–45%). F 1865 concentrations: 0.1–1 mg/liter of red blood cell suspension, i.e.: $3.26 \cdot 10^{-7}$ to $3.26 \cdot 10^{-6}$ M.

Results: two categories of sites:

high-affinity binding sites with $n_1 = 1.24 \cdot 10^{-6}$ M of packed red blood cells and $K_1 = 49.5 \cdot 10^{6}$ mol^{-1};

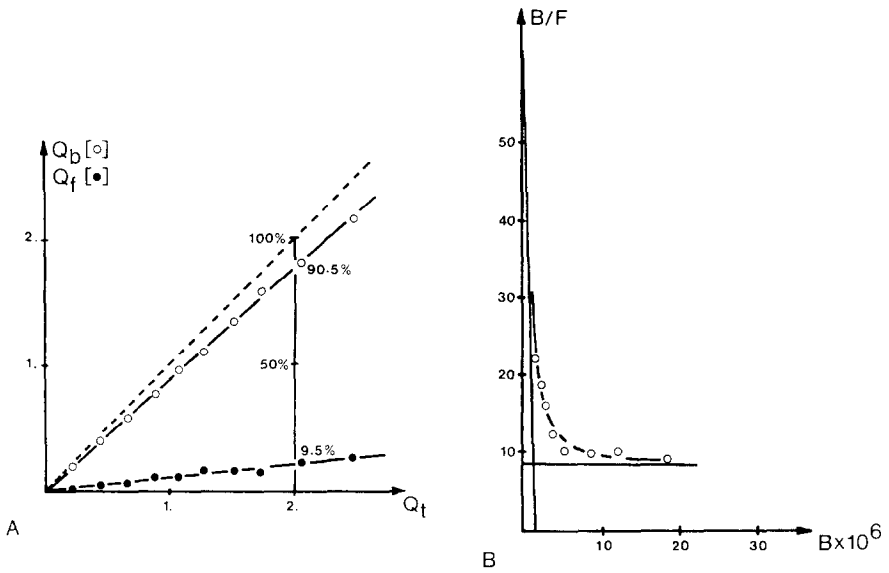

Fig. 5. Binding of F 1865 to RRBC. A: binding of F 1865 to RRBC (hematocrit 45%). *X*-axis: total concentration of F 1865 in RRBC suspension: Q_t (mg/liter); *Y*-axis: recovered quantity of F 1865 (mg) in 450 ml of packed red blood cells: Q_b; recovered quantity of F 1865 (mg) in 550 ml of buffer solution: Q_f. B: Scatchard plot of the binding of F 1865 to RRBC. *B*, mol of F 1865 bound per liter of packed red blood cells; *F*, free concentration of F 1865 (M red blood cell suspension).

low-affinity binding sites with $n_2 = 2.19 \cdot 10^{-3}$ M of packed red blood cells and $K_2 = 3.98 \cdot 10^3$ mol^{-1}.

Distribution of F 1865 in serum albumin / red blood cell system

The system used was that of the "reconstituted" blood. Concentrations of the constituents were physiological: erythrocyte suspension of 45% in a 40 g/liter solution of serum albumin.

In man (Fig. 6): 0^+ red blood cells hematocrit: 45%. HSA concentration: 40 g/liter. F 1865 concentrations: 0.027–1.6 mg/liter of red blood cell suspension, i.e. $8.76 \cdot 10^{-8}$ to $5.19 \cdot 10^{-6}$ M.
Results:
Sedimentation (Fig. 6A):
fraction bound to erythrocytes = 35%;
fraction bound to HSA + free fraction = 65%.
Dialysis (Fig. 6B):
fraction bound to erythrocytes = 35%;
fraction bound to HSA = 64%;
free fraction = 1.0%.

In the dog (Fig. 7) Red blood cell hematocrit: 45%. DSA concentration: 40 g/liter. F 1865 concentrations: 0.10–1 mg/liter of red blood cell suspension, i.e. $3.26 \cdot 10^{-7}$ to $3.26 \cdot 10^{-6}$ M.
Results by sedimentation:
fraction bound to erythrocytes: 62.5%;
fraction bound to DSA + free fraction: 37.5%.

Discussion

Two points should be emphasized before discussing the results: (1) Limitations caused by the sensitivity of assay and the poor solubility of the compound made it impossible to obtain the saturation plateau. (2) An attempt was made to evaluate binding of F 1865 to the dialysis membrane. Taking technical difficulties into account (residual liquid on membranes), it may be estimated that the bound fraction of F 1865 varied from 30% in the absence of protein to 3% in the presence of a solution of HSA at 40 g/liter. This would explain the decrease in differences between sedimentation and dialysis in the case of DRBC where binding of F 1865 was stronger than with HRBC. Similarly for distribution in the HSA-HRBC system, both methods gave identical results

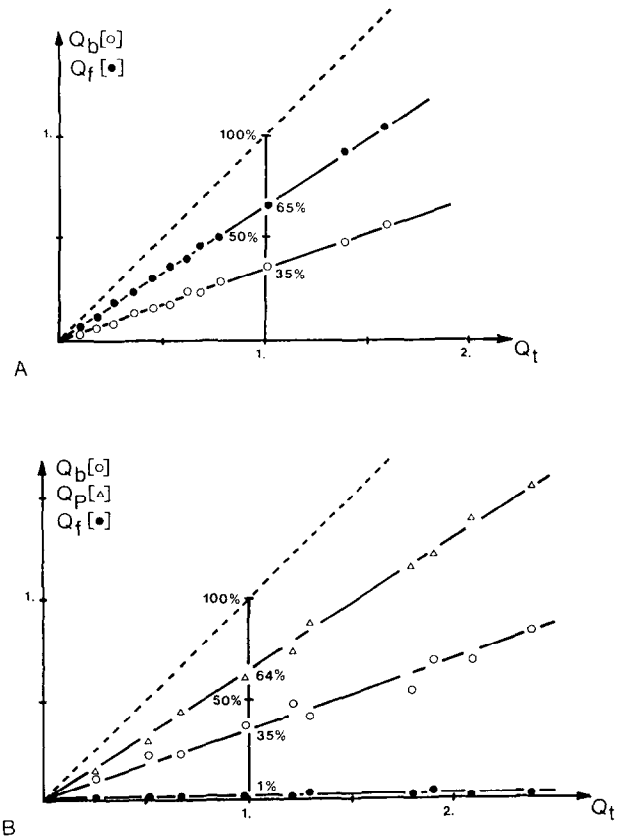

Fig. 6. Distribution of F 1865 in human reconstituted blood (HSA/HRBC system). A: sedimentation experiments. *X*-axis: total concentration of F 1865 in the HSA/HRBC system: Q_t (mg/liter). *Y*-axis: recovered quantity of F 1865 (mg) in 450 ml of packed HRBC: Q_b; recovered quantity of F 1865 (mg) in 550 of supernatant: Q_f (F 1865 free + F 1865 bound to HSA). B: equilibrium dialysis experiments. *X*-axis: total concentration of F 1865 in the HSA/HRBC system: Q_t (mg/liter). *Y*-axis: recovered quantity of F 1865 in 450 ml of packed HRBC: Q_b. Recovered quantities of F 1865 in 550 ml of HSA solution (40 g/liter): F 1865 bound to HSA Q_p, F 1865 free (free concentration measured in the buffer compartment), Q_f.

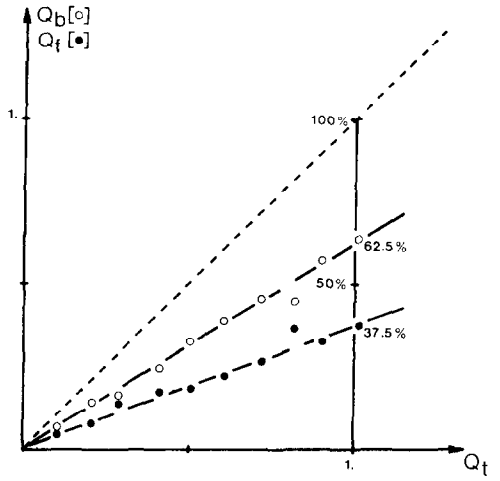

Fig. 7. Distribution of F 1865 in dog reconstituted blood (DSA/DRBC system). Sedimentation method. *X*-axis: total concentration of F 1865 in the DSA/DRBC system: Q_t (mg/liter). *Y*-axis: recovered quantity of F 1865 (mg) in 450 ml of packed DRBC: Q_b; recovered quantity of F 1865 (mg) in 550 ml of supernatant: Q_f (F 1865 free + F 1865 bound to DSA).

because of the strong binding, shifting equilibrium of membrane binding.

Results obtained show that F 1865 was bound to serum albumin more strongly in man than in the dog. Binding experiments showed a single class of binding sites on serum albumin both in man and in the dog (Figs. 1B and 2B); so the percentages of binding to serum albumin at physiological concentrations (Figs. 1A and 2A) were in direct relation to the values of binding parameters (association constants and number of sites).

For red blood cells the highest binding constant was seen in the rat, decreasing in the dog and in man. Binding experiments showed two classes of binding sites (Fig. 3B, 4B, 5B). Percentages of binding to erythrocytes at physiological concentrations (hematocrit 45%) (Fig. 3A, 4A, 5A) were very similar in man and in the dog whereas distribution between erythrocytes and serum albumin were different in man and in the dog (Figs. 6A and 7A) (binding percentages were nearly inverted).

In the case of binding to erythrocytes, the existence of two classes of binding sites made possible the measurement of identical binding percentages in two different systems (HRBC/buffer and DRBC/buffer). On the other hand, the presence of serum albumin removed ambiguity and permitted to differentiate the two systems.

Study of distributions in vitro of the compound in the red blood cell/serum albumin system at physiological concentrations clearly showed binding to red cells to be greater in the dog than in man. In such a system there are two simultaneous

and competitive equilibria, the free fraction F (in M) being in equilibrium with those fractions bound respectively to red blood cells C and to proteins P.

$$P \rightleftarrows F \rightleftarrows C$$

Considering the kinetic aspect of this phenomenon, it can easily be shown that adopting conditions of concentrations far from saturation of binding sites, Eqn. 1 is obtained providing the distribution coefficient C/P (cf. Appendix).

$$C/P = \frac{\alpha . K_{aC}}{\beta . K_{aP}} \quad (1)$$

α and β being the concentrations in terms of number of binding sites in the medium, K_{aC} and K_{aP} binding or association constants for red blood cells and serum albumin, respectively.

Applying this equation to the results obtained, very satisfactory agreement was seen between values for distribution coefficients as measured and values calculated from physico-chemical binding parameters (Table 1).

Experiments involving distribution in the serum albumin/red blood cell system can be used to confirm values of physicochemical binding parameters. This is particularly valid for binding to serum albumin, the study of which raises a considerable number of technical problems as already mentioned.

It is well known that the physicochemical study of binding of a drug to a substrate requires separation of the bound form and the free form. Whereas in the case of red cells that separation is very easy by centrifugation or filtration, by contrast study of binding to serum albumin or to any other soluble macromolecule can be carried out only by using a molecular filter, most often by equilibrium dialysis. Because of its very low solubility, the compound studied was reversibly fixed to the dialysis membrane. There was thus competitive binding of the compound between protein and the dialysis membrane which was reflected by variation in binding isotherms in accordance with the protein concentrations. To decrease this effect, ethanol was added to the medium in order to better dissolve the compound and thereby also decreasing influence of the protein concentration. Parameters concerning binding of the compound to serum albumin were thus determined in the presence of variable quantities of ethanol. Extrapolation to a nil alcohol concentration gave values of binding parameters in an ionic buffer medium. However, the variation in binding parameters in relation to ethanol concentration was not linear and the accuracy of extrapolated values was thus markedly decreased and hence the need to confirm these approximate values by in vitro studies of distribution. So, the distribution of F 1865 between serum albumin and erythrocytes in "reconstituted" blood was used to confirm the binding parameters of the product to serum albumin. Although different in the application modalities, this procedure was to be compared with the method proposed by Garrett and Lambert (1973), Garrett and Hunt (1974), Derendorf and Garrett (1983) and Almayer and Garrett (1983).

The values of binding constants of F 1865 to HSA and DSA correlated with those measured for negatively charged molecules (Tillement et al., 1984), give values ranging from 10^4 to 10^6 mol^{-1}).

Indeed physicochemical studies of stability of F 1865 in aqueous buffers show that the acidic function is regenerated rapidly, giving the carbonyl derivative. These remarks agreed also with results of binding experiments on erythrocytes showing two categories of binding sites: (1) high-affinity binding sites, few in number, corresponding to the carbonyl derivative, and (2) low-affinity binding sites, more numerous, corresponding to the unchanged product.

TABLE 1

Distribution of F 1865 in the serum albumin/red blood cell system

	Measured distribution coefficient C/P	Calculated distribution coefficient $\alpha K_{aC}/\beta K_{aP}$
Man	0.54	0.53
Dog	1.67	1.44

Last, the various techniques used clearly confirmed the difference in behaviour of F 1865 in man and in the dog. The pharmacokinetics of the compound in man and in the dog would show similar differences and a more precise interpretation of this would require more extensive investigation.

Appendix

Establishment of the expression of the distribution coefficient

Free fraction is in equilibrium with fractions bound respectively to protein (SA) and to red blood cells (RBC):

$$P \rightleftarrows SA + F \tag{1}$$

$$F + RBC \rightleftarrows C \tag{2}$$

where

F = concentration of free compound at equilibrium,

P = concentration of compound bound to SA at equilibrium,

C = concentration of compound bound to RBC at equilibrium.

Considering the kinetic aspect, the rate of association is equal to the rate of dissociation when equilibrium is achieved. Association rates are proportional to the concentration of the free compound and to the concentration of unoccupied sites. Dissociation rates are proportional to concentrations of occupied sites.

The following may be noted for binding to serum albumin:

$$\text{Association rate } v_{aP} + (k_1)_P \cdot F \cdot p_s \tag{3}$$

p_s being the concentration of non-occupied protein sites, with:

$$p_s = \beta - P \tag{4}$$

β being the total concentration of protein binding sites, P being the concentration of occupied sites (equal to the concentration of the compound bound to serum albumin).

$$\text{Dissociation rate: } v_{dP} = (k_{-1})_P \cdot P \tag{5}$$

The same equations exist for binding to red cells:

$$\text{Association rate: } v_{aC} = (k_1)_C \cdot F \cdot c_s$$

c_s is the concentration of non occupied red cell sites, with $c_s = \gamma - C$, γ is the total concentration of red cell binding sites. C is the concentration of occupied sites (equal to the concentration of the compound bound to red cells).

$$\text{Dissociation rate: } v_{dC} = (k_{-1})_C \cdot C$$

In both cases and at steady state the following apply:

$$|v_{aP}| = |v_{dP}| \quad \text{and} \quad |v_{aC}| = |v_{dC}|$$

The following may be noted from the above expressions:

$$\text{for serum albumin} \quad \frac{(k_1)_P}{(k_{-1})_P} \cdot F = \frac{P}{\beta - P}$$

$$\text{or} \quad K_{aP} \cdot F = \frac{P/\beta}{1 - P/\beta}$$

for red cells

$$\frac{(k_1)_P}{(k_{-1})_C} \cdot F = \frac{C}{\gamma - C} \quad \text{or} \quad K_{aC} \cdot F = \frac{C/\gamma}{1 - C/\gamma}$$

K_{aP} and K_{aC} are the association equilibrium constants for the equilibrium (1) and (2). Equalizing F, the following is obtained:

$$\frac{P/\beta}{K_{aP}(1 - P/\beta)} = \frac{C/\gamma}{K_{aC}(1 - C/\gamma)}$$

If the situation is far from saturation in the two equilibria, i.e. $P \ll \beta$ and $C \ll \gamma$, the following is obtained:

$$\frac{C}{P} = \frac{\gamma \cdot K_{aC}}{\beta \cdot K_{aP}}$$

Distribution experiments in the RBC/SA system at physiological concentrations showed free fraction of F 1865 to be negligible (about 1%). So,

the concentration of F 1865 bound to SA was taken as equal to the concentration of F 1865 in the supernate ($P \neq P + F$).

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