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"In vitro" model for the evaluation of drug distribution and plasma protein-binding relationships

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Summary

A pharmacokinetic model for drug distribution in vitro, in the presence of bovine serum albumin (BSA) was developed. It consists of a 3-phase ($w_1/o/w_2$) partition apparatus, where the first aqueous phase w_1 represents the plasma compartment, the octanolic and the second aqueous phase w_2 represents hydrophobic and hydrophilic tissues together. The drugs under examination (carbamazepine, denzimol, naproxen and ketoprofen) were introduced in the first aqueous phase and their decrease as a function of time was followed by HPLC. The obtained data (R) were expressed as the ratio between the equilibrium distribution of drugs in the absence and in the presence of BSA. Results were consistent with the known behaviour of the drugs studied "in vivo", and contribute to evaluate the influence of drug protein-binding on the body disposition.

Introduction

Plasma protein binding can be considered a mode of transport for most drugs; in special cases, however, it can also modify their tissue distribution and their pharmacokinetic behaviour. Nevertheless, traditional evaluations of drug protein binding by equilibrium dialysis, ultrafiltration or other "in vitro" techniques do not account for its role in the picture of drug disposition "in vivo". In fact, the binding percentage obtained by "in vitro" determinations is just a function of the relative concentrations of protein and ligand under limited experimental conditions, whereas, in physiological conditions, a number of other equilibria, first of all passive diffusion through lipophilic tissues, can enter into competition. On the other hand, the association constant NK provides more accurate information about the mechanism of binding, but it is conditioned by the knowledge of the number and the nature of the binding sites. Thus, it is frequently necessary to perform elaborate calculation programs which may not be generally applicable, because the binding of most drugs to plasma proteins is non-specific and represents the overall binding at different sites.

This study is an attempt to propose a general method in order to evaluate protein-binding "in

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vitro" in the presence of different phases simulating tissues.

The experimental model presented in this paper was developed on the basis of the assumption that the distribution of drugs "in vivo" is ruled by the passive diffusion law, and that the affinity of substances to lipophilic tissues (defined by oil/water partition coefficient) plays a major role in their transport, distribution and action. A three-phase partition apparatus (w/o/w), previously proposed by Rodriguez et al. (1984) for evaluating the "in vitro" absorption of drugs, was modified to fit the purpose of the present study. In the experiments carried out with this modified model, the 3 phases simulate blood and lipophilic/hydrophilic tissue compartments.

A buffered solution of bovine serum albumin (BSA) was used to simulate the plasma compartment, because drug binding occurs to this protein in most cases. *n*-Octanol was used as a solvent of choice for simulating lipophilic tissues, as demonstrated by the research of Leo and Hansch (1971). Two anti-inflammatory and two anticonvulsant substances were used as model drugs; these drugs were chosen because they represent both a wide range in chemical–pharmacokinetic and in oil–water solubility characteristics, even though all of them are strongly bound to plasma proteins.

Materials and Methods

The HPLC instrument used was P. Elmer, Series 2; Detector UV, LC75 P. Elmer. Operating conditions: stationary phase, Lichrocart–Lichrospher column 250-4, 100 RP8,5 μ Merck. Flow rates 1–1.5 ml/min. Crystallized bovine serum albumin (BSA) was purchased by Sigma. *n*-Octanol was Merck, samples analytical grade.

The drug samples were kindly given from Recordati Pharmaceutical Industry, Milan.

Determination of binding properties of BSA pretreated with octanol

20 ml of phosphate buffer pH 7.4 0.06 M, containing NaCl 0.9% and BSA 4.5% was saturated with octanol, then ultrafiltrated through a membrane Amicon T 10 PM 10, and successively diluted with phosphate buffer, until no more octanol was present in the ultrafiltrate (monitoring of n-octanol in the ultrafiltrate was carried out by gas chromatography). The resulting solution was adjusted for the original volume and used for protein-binding determinations of carbamazepine and ketoprofen, according to the method already described (Dal Pozzo et al., 1987), in comparison with another solution prepared with native (untreated) BSA. The two samples, containing respectively the octanol-treated and untreated BSA, finally had the same binding with carbamazepine (72%) and ketoprofen (95%).

Description of the apparatus

The apparatus showed in Fig. 1 consists of two glass cylinders connected by isoversinic tubes. The volume of the reservoir R was made variable by moving the piston P_1 upwards after every drawing of liquid. The filter F was introduced in order to capture possible microbubbles, and consists of a small tube filled by glass-wool.

The aqueous phases w_1 and w_2 were aqueous solutions of phosphate buffer pH 7.4, 0.06 M and NaCl 0.9%. The oil phases o_1 and o_2 were *n*-octanol. Before use, the different phases were saturated with each other. The peristaltic pump P assures the constant flow rate through cells and tubes of 11 ml/min.

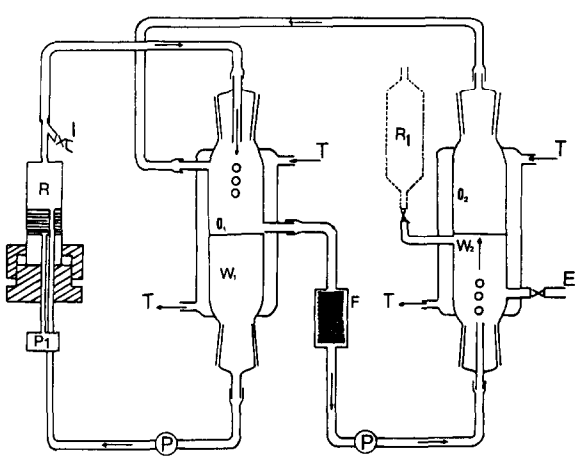

Fig. 1. R, R_1, reservoirs; O_1, O_2, oil phases; W_1, W_2, aqueous phases; P, peristaltic pump; P_1, piston; T, thermostatic jacket; F, filter; I, E, by-passes.

TABLE 1

Operating conditions for the determination of drugs

Drug	Extraction				λ (nm)	Retention time
	Solvent	pH	I.S.	Mobile phase		
Naproxen	dichloromethane	1	6-methoxy-2-naphtyl-acetic acid	52% acetonitrile in 0.01 M/ phosphoric acid pH 2.5	230	5.9′ (I.S. 5.1′)
Ketoprofen	dichloramethane	1	benzoyl-propionic acid	52% acetonitrile in 0.01 M/ phosphoric acid pH 2.5	248	6.3′ (I.S. 3.0′)
Carbamazepine	chloroform	10	denzimol	53% acetonitrile in 0.01 M/ phosphate buffer pH 7.4	220	2.6′ (I.S. 4.6′)
Denzimol	chloroform	10	carbamazepine	53% acetonitrile in 0.01 M/ phosphate buffer pH 7.4	220	4.6′ (I.S. 2.6′)

Procedures for use of the apparatus and drugs assay

Of a solution of the sample drug (2 mg/ml of ethanol) 400 μl were added to 40 ml of the aqueous phase and introduced in the reservoir R and in the compartment W_1 of the apparatus. When experiments were carried out in the presence of BSA, this phase contained also 4.5% of the protein. In succession, the compartment W_2 and the reservoir R_1 were filled with simple buffer solution and the compartments O_1 and O_2 with the *n*-octanol phase, with accurate exclusion of air bubbles. At time intervals, 2.5 ml of the aqueous phase were drawn, either from W_1 using the by-pass I, or from W_2 using the by-pass E.

After centrifugation, 2 ml of each sample were introduced in separatory funnels, together with a suitable quantity of internal standard I.S. and the pH was adjusted.

The aqueous solutions were extracted three times with organic solvent (10,5 and 5 ml). The organic phases were dehydrated by filtration through a paper 1PS Whatmann, and evaporated to dryness under N_2 atmosphere, at 40 °C. The residues, dissolved with suitable quantities of methanol, were analyzed by HPLC. More experimental details for the single drugs are given in Table 1.

Results and Discussion

In the experiments carried out with the 3-phase partition apparatus represented in Fig. 1, the distribution of drugs in the presence of BSA and lipophilic/hydrophilic phases simulating tissues was studied. There was no evidence of protein denaturation, under the described experimental conditions, even after prolonged contact between the BSA and the octanolic phase. Moreover, the octanol-treated albumin showed to have the same binding properties toward drugs as untreated samples. In fact, two drugs, binding to quite different sites of albumin, when submitted to equilibrium

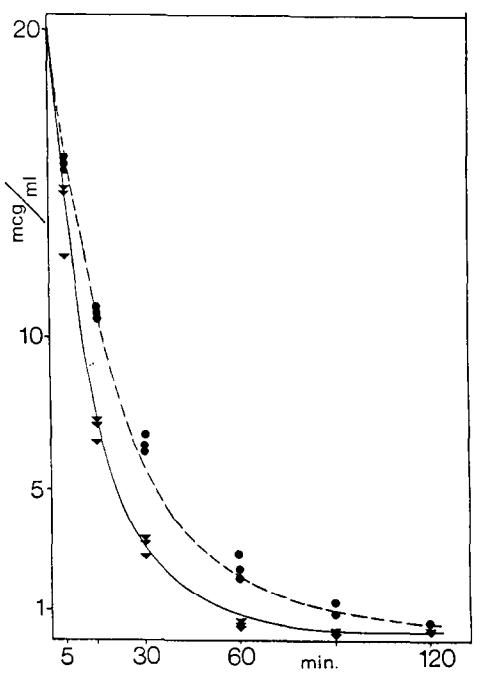

Fig. 2. Distribution profile of carbamazepine, determined in the releasing phase w_1, in the absence (continuous line) and in the presence (dotted line) of BSA 4.5%, as a function of time.

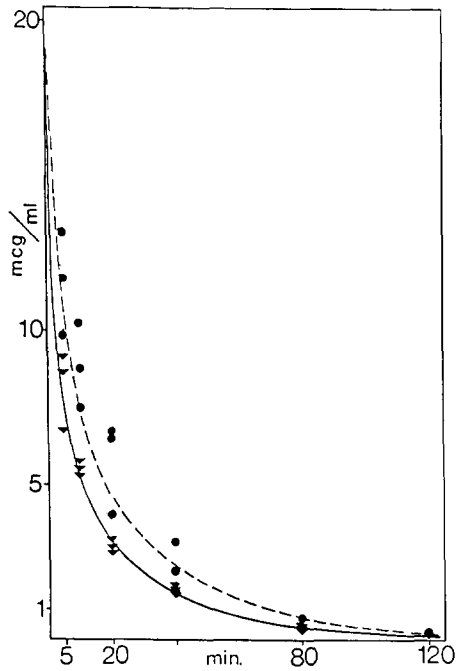

Fig. 3. Distribution profile of denzimol, determined in the releasing phase w_1, in the absence (continuous line) and in the presence (dotted line) of BSA 4.5%, as a function of time.

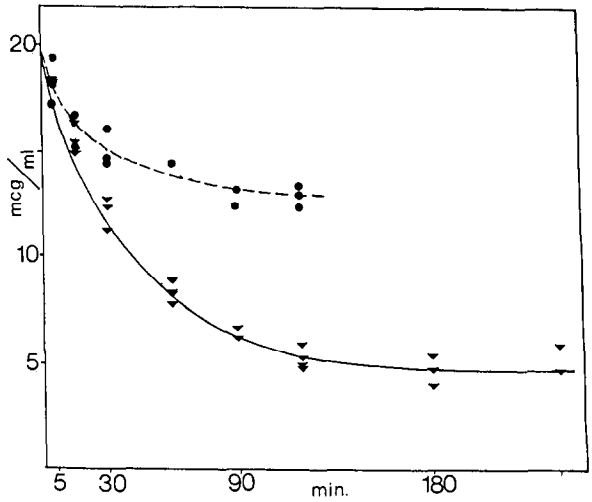

Fig. 4. Distribution profile of ketoprofen, determined in the releasing phase w_1, in the absence (continuous line) and in the presence (dotted line) of BSA 4.5%, as a function of time.

dialysis with the protein gave the same results, either before or after the octanol treatment. Figs. 2–5 represent the distribution profiles of the 4 drugs examined, determined following their disappearance in the releasing phase w_1, in the absence and in the presence of BSA, vs time. The determination of the drugs in the receiving phase w_2, described in the preliminary paper (Dal Pozzo et al., 1987), proved to be of no general application, i.e., in the case of highly hydrophobic drugs; in fact, the latter tend to accumulate in the octanolic phase, and their amount in the receiving phase w_2 is too low, at least for the sensitivity of the analytical method employed.

In Figs. 2 and 3, it appears that the distributions of carbamazepine and denzimol are practically unaffected by the presence of the protein, at the equilibrium. On the contrary, ketoprofen and naproxen distributions (see Figs. 4 and 5) change dramatically when BSA is present, so that they become extremely slow. In an attempt to define the relationship between protein binding and distribution in plasma and tissues, we expressed by R the ratio between the concentration of each drug in the absence of protein and its concentration in

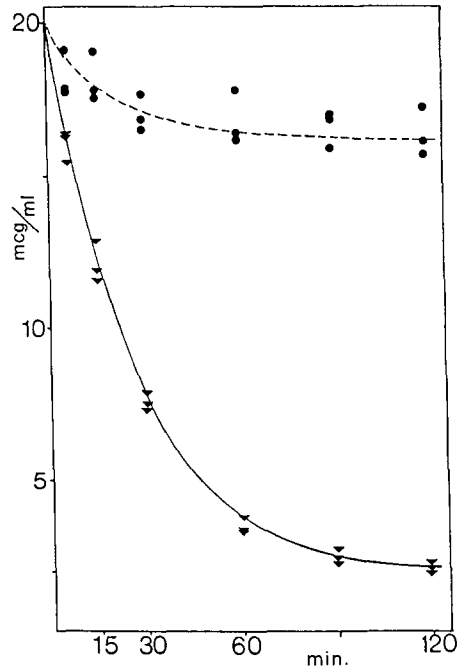

Fig. 5. Distribution profile of naproxen, determined in the releasing phase w_1, in the absence (continuous line) and in the presence (dotted line) of BSA 4.5%, as a function of time.

TABLE 2

Pharmacokinetic data for the drugs investigated

Substance	PB (%)	NK (10^{-3})	V_d (liters)	log P	R
Naproxen	99.95	6500	11.2	0.28	5.1
Ketoprofen	95.3	–	7.7	−1	2.13
Carbamazepine	73.7	3.5	70	2.45	1.02
Denzimol	93	30	250	3.4	1

PB, protein binding; NK = association constant; V_d = distribution volume; log P = log C_{oct}/C_{H_2O} = partition coefficient; R = $C^0 - C^\infty/C^0_{ALB.} - C^\infty_{ALB.}$ = ratio, at equilibrium, between distributed substance in the absence and in the presence of BSA, where C^0 = initial concentration in the phase W_1; C^∞ = Final concentration in the phase W_1; $C^0_{ALB.}$, $C^\infty_{ALB.}$, initial and final concentrations in the phase W_1 containing BSA 4.5%.

the presence of protein, determined in the first aqueous phase W_1, at the equilibrium. When R is equal or very close to unity, one may assume that the protein binding does not influence significantly the body disposition of the drugs.

In Table 2, some pharmacokinetic parameters related to the 4 drugs and taken from literature values (Goodman and Gilman, 1985; Dal Pozzo et al., 1983) are reported, in parallel with their values of R; we may observe that the protein binding values (PB) are generally high for the 4 drugs, whereas the distribution values (V_d) are significantly different (from 7.7 up to 250 liters). The lack of correlation between the two parameters means that a high protein binding does not necessarily influence the distribution of drugs. On the other hand, the better correlation observed between V_d and both log P and R values points out the competition between protein binding and affinity to lipophilic tissues on the drug distribution. Moreover, a relationship between the distribution profiles of drugs in the absence and in the presence of albumin expressed by R are consistent with the known binding mechanisms of the two groups of drugs investigated: in the case of the two anticonvulsants, having a low-affinity, high-capacity binding to albumin, that does not affect their distribution through octanol, the R value is 1; whereas, in the case of the two anti-inflammatories, having a high-affinity, low-capacity binding to the protein that is able to compete with the distribution to octanol or other lipohilic phases, R values are respectively 2 and 5.

Conclusions

The experimental data obtained from the apparatus described herein provide a better understanding of the implication of the binding to albumin in the pharmacokinetic picture of drugs than simple binding measurements performed by traditional methods. This may be a useful predictive tool when protein-binding and distribution aspects are important criteria of choice for members of large series submitted to preliminary screenings, as, for instance, in the case of antibiotics.

Generally speaking, evaluations of the drugs' behaviour from alternative pharmacokinetic models in vitro can be considered when new compounds are being developed, reducing, as a consequence, the number of animal experiments.

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