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Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood

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The distribution of cyclosporin A between plasma, leucocytes and erythrocytes was studied in vitro by means of sedimentation in Ficoll-Paque and dextran. The uptake by erythrocytes was found to be about 50% and the fraction of cyclosporin A bound to leucocytes amounted to 15%. Fractionation of plasma by ultracentrifugation also showed that two thirds of the drug were associated with lipoproteins whereas binding studies with isolated lipoproteins and plasma also indicated that lipoproteins were the major complexing constituents for cyclosporin A in plasma. The binding of cyclosporin A to erythrocytes and lipoproteins seems to be a linear process. The binding to the leucocytes may be a saturable process, however it is of minor importance in terms of the overall binding capacity in the blood.

Cyclosporin A, a cyclic endecapeptide, has been shown to be a powerful immunosuppressant in different animal species and in man (Borel et al 1977). In view of its hydrophobic character, cyclosporin A would be expected to interact extensively with blood lipid constituents. In this work we report the in vitro distribution of cyclosporin A between plasma, leucocytes and erythrocytes of human and rat blood. By using the technique of ultracentrifugation we also identified the most important plasma binding fraction as lipoproteins.

MATERIALS AND METHODS

Chemicals

[^{3}H]Cyclosporin A (79·3 μCi mg^{-1}) was synthesized at the Pharmaceutical Department of Sandoz Ltd, Basle. The radiochemical purity of the labelled compound was 98%.

Preparation of samples

Freshly heparinized blood was collected from fasting Sprague Dawley rats and from fasting normal individuals (Blood Bank, Basle) and plasma was obtained by centrifugation at 400 *g* for 5 min.

The lipoproteins were prepared as a whole (very low, low, high density—VLDL, LDL, HDL) by ultracentrifugation (Sager et al 1979). Potassium bromide was added to human plasma to achieve a density of 1·195 g ml^{-1} and then ultracentrifuged for 45 h at 105 000 *g* at 4 °C. The floating lipoproteins were withdrawn and dialysed against 5 changes of a 4 times larger volume phosphate buffer 0·02 M pH 7·4 at 4 °C for 24 h. The protein content of this lipoprotein mixture was determined (Lowry et al 1951) and adjusted to 2 g litre^{-1} approximate to physiological concentrations (Hatch 1968).

[^{3}H]Cyclosporin A, 1 mg, was dissolved in 20 ml ethanol; 100, 50, 20, 10 and 5 μl of this solution were then added to 10 ml of blood, plasma or lipoproteins solution to give the concentrations 500, 250, 100, 50 and 25 ng ml^{-1}. To avoid a haemolytic alteration of the erythrocytes, the blood was centrifuged to isolate the red blood cells and the ethanol aliquots were added to the plasma, the latter was then added to the erythrocytes to reconstitute the blood.

Distribution in whole blood

In a preliminary experiment, the time course to equilibration between plasma and blood cells was studied. [^{3}H]Cyclosporin A was added to human blood (see above) and warmed to 37 °C, then the mixture was swirled gently in a water bath. Aliquots were withdrawn at 0·33, 0·66, 1, 2, 4, 6 and 8 h and centrifuged to separate plasma. Erythrocytes and plasma samples (100 mg) were placed on filter pads and dried in an oven at 80 °C, plasma for 20 and erythrocytes for 45 min. The samples were then combusted in a Packard Tri-Carb sample oxidizer and the radioactivity measured by scintillation counting.

Whole blood (from rat and man) containing varying concentrations of [^{3}H]cyclosporin A was incubated for 1 h at 37 °C with gentle swirling. The isolation of leucocytes and erythrocytes was a modification from a previously described method (Nessi & Frei 1977). To 2 ml of incubated blood was added the same volume of a balanced salt solution (1

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volume solution A: anhydrous D-glucose 0·1% $CaCl_2 \cdot 2H_2O$ 5×10^{-5} M, $MgCl_2 \cdot 6H_2O$ $9{\cdot}8 \times 10^{-4}$ M, KCl $5{\cdot}4 \times 10^{-3}$ M, Tris 0·145 M with 9 volumes solution B: NaCl 0·14 M). The diluted blood samples (4 ml) was carefully layered on 3 ml Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The samples were then centrifuged at 400 *g* for 35 min at 20 °C. At the top of the tube the diluted plasma was first withdrawn (Fraction I, 3 ml); then the small lymphocytes layer was collected with the Ficoll-Paque layer (Fraction II, 2·8 ml). At the bottom of the tube the granulocytes and the erythrocytes were resuspended in 0·9% NaCl (the volume of the saline solution was identical to that of the diluted plasma). 3 ml of this suspension were poured into a polyethylene tube inclined at 45 °C and containing 3 ml of 15% Dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0·9% NaCl. The tubes were left for 3·5 h at room temperature. The clear supernatant which contains the granulocytes was collected (Fraction III, 5·2 ml); the bottom of the tube (Fraction IV, 0·8 ml) consisted of erythrocytes. Drug concentrations and cell counts were determined by liquid scintillation counting and electronic particle counting (Coulter counter Model S) in each fraction. In all experiments over 90% of the cells and the radioactivity that was in the original blood sample could be accounted for in the various fractions. The percent of [^{3}H]cyclosporin A in each fraction was calculated as the amount of the radioactivity present in this fraction divided by the total amount of radioactivity present in the 4 fractions.

Distribution in plasma

To plasma samples containing [^{3}H]cyclosporin A at various concentrations was added potassium bromide to achieve a density of 1·195 g ml^{-1}; the samples were then centrifuged for 45 h at 105 000 *g* at 4 °C. At the end of the run, 13 fractions of about 0·7 ml were withdrawn with a syringe from the top to the bottom of each tube. Radioactivity and protein concentrations were determined in each fraction by liquid scintillation counting and by the Biuret method. The floating lipoproteins corresponding to the three upper layers were separated from the other plasma proteins corresponding to the nine lower layers by a thin protein-free water phase. The percent of [^{3}H]cyclosporin A in the lipoprotein fractions was calculated as the radioactivity present in the three first fractions divided by the total radioactivity present in the entire sample; likewise the percent of [^{3}H]cyclosporin A in the residue fractions (albumin, globulins, etc.) was calculated as the radioactivity in the nine last fractions divided by the total radioactivity present in the 13 fractions. Additionally the percent [^{3}H]cyclosporin A free was calculated as the concentrations in the protein-free fraction divided by the concentrations in the sample before ultracentrifugation.

Plasma protein binding experiments

Cyclosporin A was found to be essentially insoluble in aqueous buffer so that the ultracentrifugation method was preferred to the conventional method of equilibrium dialysis. The plasma and lipoproteins samples spiked with different amounts of [^{3}H]cyclosporin A were centrifuged at 20 ° or 4 °C for 14 h at 150 000 *g*. The fraction of substance bound was calculated from the concentration in the protein-free supernatant (unbound drug) and in the original solution (total drug). Using a protein-free solution it was determined, by measuring the concentration of the drug at various levels in the centrifuged tube, that no sedimentation of drug occurred.

RESULTS

Distribution of [^{3}H]cyclosporin A in blood

After addition of [^{3}H]cyclosporin A to human blood, equilibration of drug between plasma and erythrocytes occurred rapidly: steady state was established during the first 20 min of incubation. The distribution ratio between erythrocytes and plasma remained constant (~1·5) in the concentration range studied.

[^{3}H]Cyclosporin A was incubated for 1 h with blood and the mixture was fractionated by means of Ficoll-Paque and dextran as described in 'Materials and Methods'. The percentage of leucocytes and erythrocytes in the different fractions is given in Table 1. Fractions II and III account for 60–80% of the leucocytes, whereas fraction IV accounts for 85% of the erythrocytes, the degree of contamination of leucocytes by erythrocytes, and conversely, is

Table 1. Isolation of leucocytes and erythrocytes in human and rat blood components by means of Ficoll Paque and dextran. The percentages of the total number of white and red cells in each fraction are given.

	Species			
	Man		Rat	
Fraction	Leucocytes ($5{\cdot}9 \times 10^9$ litre^{-1})	Erythrocytes ($4{\cdot}0 \times 10^{12}$ litre^{-1})	Leucocytes ($7{\cdot}8 \times 10^9$ litre^{-1})	Erythrocytes ($6{\cdot}5 \times 10^{12}$ litre^{-1})
Plasma (I)	20	6	13	6
Lymphocytes (II)	32	1	59	1
Granulocytes (III)	30	9	20	8
Erythrocytes (IV)	18	84	8	85

relatively low. Results in both species were similar: 10 to 20% of the drug amount were found in the leucocyte fractions, 40 to 50% were in the erythrocyte fraction, and 30 to 40% in the plasma fraction (Table 2).

Table 2. Distribution of [^{3}H]cyclosporin A in rat and human blood as function of blood level. The percentages of total drug mass in each fraction is given.

Fraction	Plasma (I)		Lympho-cytes (II)		Granulo-cytes (III)		Erythro-cytes (IV)	
blood concn (ng ml^{-1})	Man	Rat	Man	Rat	Man	Rat	Man	Rat
500	33	42	4	7	5	7	58	44
250	32	42	6	8	5	5	56	45
100	35	40	7	10	6	7	53	43
50	47	37	5	10	6	11	41	42
25	33	36	9	10	12	16	45	38

The distribution ratios erythrocyte-leucocyte E:L and plasma-leucocyte P:L decreased as the blood concentrations decreased (Table 3). However these observations should be restrained by the fact that only the decrease of the ratio E:L in man is significantly correlated ($P<0{\cdot}05$) with the decrease of the corresponding blood concentrations. On the contrary, the distribution ratio erythrocyte-plasma was independent of [^{3}H]cyclosporin A concentration (Table 3). Thus the blood distribution of [^{3}H]cyclosporin A may be a composite of two processes in the concentration range studied: a linear distribution process with plasma proteins and erythrocytes, and a non-linear process with leucocytes.

Table 3. Distribution ratios erythrocyte-leucocyte (E:L), plasma-leucocyte (P:L) and erythrocyte-plasma (E:P) as function of blood level.

	Distribution ratio					
	E:L		P:L		E:P	
blood concn (ng ml^{-1})	Man	Rat	Man	Rat	Man	Rat
500	6·4	3·1	3·7	3·0	1·8	1·1
250	5·1	3·5	2·9	3·2	1·8	1·1
100	4·1	2·5	2·7	2·3	1·5	1·1
50	3·7	2·0	4·3	1·8	0·9	0·9
25	2·1	1·5	1·6	1·4	1·4	1·1

Distribution of [^{3}H]cyclosporin A in human plasma

The profile of the distribution of [^{3}H]cyclosporin A in human plasma is presented in Fig. 1: the initial peak of radioactivity (fractions 1, 2, 3) coincided with the protein peak containing the lipoproteins.

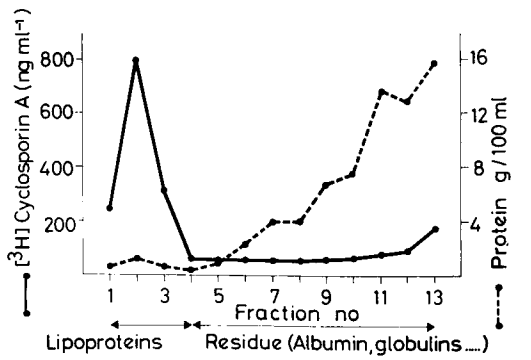

FIG. 1. Ultracentrifugation pattern of human plasma proteins and distribution of [^{3}H]cyclosporin A (250 ng ml^{-1}) in human plasma.

Table 4. Distribution of [^{3}H]cyclosporin A in human plasma as function of plasma level. The percentages of total drug mass in each fraction are given.

	Plasma concn (ng ml^{-1})				
	500	250	100	50	25
Lipoproteins	71	64	66	64	65
Residue (albumin, globulins, . . .)	25	34	31	32	32

Table 4 shows that the distribution of [^{3}H]cyclosporin A between the different plasma proteins was concentration independent. Approximately two-thirds of [^{3}H]cyclosporin A that was added to human plasma was found to be distributed among the lipoprotein fraction; on the other hand little [^{3}H]cyclosporin A was associated with the residue fraction which contained most of the plasma proteins (albumin, globulins, . . .). The bound fraction [^{3}H]cyclosporin A calculated from the concentration in the protein-free fraction was 76% independently from the drug concentration (Table 5).

Table 5. Binding of [^{3}H]cyclosporin A to human and rat plasma proteins at 4 $^{\delta}$ and 20 °C. The percentages of drug bound are given.

		Drug concns (ng ml^{-1})				
		500	250	100	50	25
		Drug bound (%)				
	Human lipoproteins	50	—	53	—	53
4 °C	Human plasma	67	68	68	71	73
	Human plasma	71	76	76	77	76
	Human plasma	89	89	—	—	87
20 °C	Human serum	93	93	—	—	93
	Rat plasma	95	95	—	—	96

* Percentages bound obtained from the plasma distribution experiments.

Binding to rat and human plasma proteins

At 4 °C the percentages bound in plasma (70%) were very near to those obtained in the distribution experiments. The comparison of the bound fraction in human plasma with the bound fraction in the lipoprotein solution (53%) suggests that the lipoproteins are the major complexing constituents for [^{3}H]cyclosporin A in human plasma (Table 5).

At 20 °C the protein binding of [^{3}H]cyclosporin A is higher (90 to 95%) than those found at 4 °C. Likewise at 4 ° and 20 °C the binding is independent of drug concentration over the range studied.

DISCUSSION

From the distribution experiments in human blood and human plasma it can be concluded that, at the therapeutic blood concentration of 500 ng ml^{-1} (Beveridge et al 1981), cyclosporin A is essentially taken up by the erythrocytes (58%), whereas the fraction of drug bound to leucocytes amounts to 9%. The fraction present in plasma consists in 4% free in plasma water, 21% bound to lipoproteins and 8% bound to other plasma proteins.

A number of drugs have been reported in recent years to bind to lipoproteins: nortriptyline (Tillement et al 1974), quinidine (Nilsen 1976), chlorpromazine (Bickel 1975), reserpine (Chen & Danon 1979), imipramine (Danon & Chen 1979), Δ^9-tetrahydrocannabinol (Klausner et al 1975), probucol (Beaumont et al 1980); it seems however that the binding of cyclosporin A to human lipoproteins is higher than most of the different bindings reported. The present results show that cyclosporin A interacts with lipoproteins independent of drug concentration; one can particularly expect that cyclosporin A enters the hydrophobic core regions of lipoproteins where it may be distributed according to its high octanol–water partition coefficient of 120. This binding of cyclosporin A to lipoproteins could have some practical consequences: the binding to lipoproteins can vary considerably with the degree of lipoproteinaemia. As cyclosporin A is 90% bound to plasma proteins and predominantly to lipoproteins, such shifts in binding may be of kinetic and clinical significance (Tillement et al 1978).

The erythrocytes represent the main binding component for cyclosporin A in human and rat blood. The distribution ratio erythrocyte-plasma was essentially constant (~1·5) over the therapeutic concentration range suggesting that the uptake by red cells obeys Nernst's law of distribution. The high lipid solubility of cyclosporin A explains its rapid uptake by erythrocytes, the nature of the binding components in erythrocytes, i.e. cell membrane, haemoglobin, carbonic anhydrase should be demonstrated in further experiments. The lower uptake of cyclosporin A by the erythrocytes at low concentrations may be attributed to a binding with other blood components which will compete with drug available to the erythrocytes. In fact, Tables 2 and 3 show that leucocytes also bind cyclosporin A: this binding seems to be saturated at concentrations higher than 100 ng ml^{-1}. A specific binding of cyclosporin A to mouse lymphocytes was effectively demonstrated (Ryffel et al 1980). Furthermore these authors could establish a correlation between the specific binding to T-lymphocytes and the preferential biological effect on these cells in in vitro systems, i.e. the inhibition of mitogen (Con A) stimulated lymphocyte cultures. From a pharmacokinetic standpoint relative to the capacity of the erythrocytes to interact with cyclosporin A, the binding capacity of the leucocytes is small. Thus, leucocytes binding would probably not influence the pharmacokinetic properties of cyclosporin A.

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