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MULTIPLE HUMAN SERUM BINDING OF TWO THIENOPYRIDINIC DERIVATIVES, TICLOPIDINE AND PCR 2362, AND THEIR DISTRIBUTION BETWEEN HSA, α_1-ACID GLYCOPROTEIN AND LIPOPROTEINS*

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Abstract—The binding of two drugs, ticlopidine and PCR 2362, chemically related to thienopyridin, potent antiaggregant agents, was studied *in vitro* to serum and to the corresponding isolated proteins, HSA, α_1-AGP, VLDL, LDL and HDL, using equilibrium dialysis at pH 7.4 and 37°. The binding of these drugs to HSA and lipoproteins was non-saturable. The binding capacity of the lipoproteins was much greater than that of HSA and appeared to be dependent on lipid content. The binding capacities of the apoproteins were less than 10% of that observed for the native lipoproteins suggesting that drug–lipoprotein binding involves drug solubilisation in the lipid phase of lipoproteins rather than a classical binding to definite sites. However drug binding to α_1-AGP was saturable with $n = 3$ for both and $K = 89{,}000$ and 33,000 for ticlopidine and PCR 2362, respectively. At physiological concentration, α_1-AGP binding capacity represented 15% of total serum binding capacity which could double in pathological states, in which the level of this protein is increased.

As a general rule, human serum albumin (HSA) is considered to be the main and often the only one binding protein for weakly acidic drugs. On the other hand, the serum binding of weak basic drugs cannot be explained solely in terms of binding to HSA [1, 2]. As reported by Borga *et al.* [3] and Piafsky *et al.* [4], many basic drugs are bound to α_1-acid glycoprotein (α_1-AGP). Furthermore, several authors have also previously shown that some basic drugs, e.g. Δ1-tetrahydrocannabinol [5], tetracycline [6], imipramine and apparented tricyclic antidepressants [7], clioquinol [8], quinidine [9], reserpine [10] and propranolol [11], are also bound to serum lipoproteins. For some of these drugs it has been clearly demonstrated that binding occurs to the three main plasma lipoproteins, VLDL, LDL and HDL.

Since some of these drugs are highly lipophilic, it seemed of interest to determine whether binding to lipoproteins is observed with other lipophilic, basic drugs and can therefore be considered as a general phenomenon. For this purpose, two thienopyridinic derivatives, ticlopidine and PCR 2362, used as potent platelet antiaggregant agents were selected. These two basic drugs have p*Ka*'s of 7.1 and 6.1, and partition coefficients of 99.2 and 99.0 in heptane/buffer system, respectively. Their binding to the different isolated lipoproteins, HSA and to α_1-AGP has been compared and the distribution of the bound fraction of their drugs among the different serum proteins has been estimated.

* Part of this work was presented at a symposium on drug–protein binding in Stockholm, March 1980.

MATERIALS AND METHODS

Materials

Human serum albumin. HSA (Sigma Chemical Co., London, U.K.; A 1887, purity 99%, FFA molar ratio = 0.04) was used, dissolved in phosphate buffer M/15 at pH 7.4.When a pool of human serum was used, HSA concentration was estimated by the bromocresol green method [12].

α_1-Acid glycoprotein. α_1-AGP (Behring; purity 99%) was used in phosphate buffer M/15 at pH 7.4. α_1-AGP concentration in serum was measured by radial immunodiffusion on plates (M-Partigen, Behringwerke).

Lipoproteins. VLDL, LDL and HDL concentrations in serum corresponding to apolipoproteins B (VLDL + LDL) and A (HDL) were measured by radial immunodiffusion on plates (M-Partigen, Behringwerke). Each isolated lipoprotein was obtained by ultracentrifugation from pooled normo-lipidemic human serum. No chylomicrons were present.

Ultracentrifugation was performed at 60,000 rpm for 2 hr at 10° using a TV 865 B vertical rotor in Sorvall model OTD 2 ultracentrifuge. Serum was made 4 M in NaCl and a discontinuous NaCl/sucrose density gradient was formed using Nelson's method [13]. In each tube the following solutions were added from bottom to top: 48% sucrose (3.5 ml), 4 M serum (5.0 ml), and 0.67 M NaCl with 0.05% EDTA (13.5 ml). And the end of ultracentrifugation, VLD, LDL and HDL were fractionated, taken out and centrifuged separately again. Each fraction was brought to its respective density with 9 g/l NaCl (*d*: 1.006) for VLDL, 95.7 g/l NaCl (*d*: 1.063) for LDL

and 513 g/l KBr (*d*: 1.350) for HDL. The ultracentrifugation was run again during 16 hr for VLDL and LDL and 40 hr for HDL; the supernatants contained the pure fraction of the different lipoproteins. VLDL, LDL and HDL were dialysed separately against phosphate buffer M/15, 9 g/l NaCl at pH 7.4 overnight. The purity of each fraction was tested by Ouchterlony's method [14] and estimated to be higher than 95%. Concentration of each lipoprotein was measured by Lowry's method [15].

Apolipoproteins. Each lipoprotein was delipidated twice by ethanol/diethylether (3:1, v/v) mixture, then three times by diethylether. Solvent to lipoprotein ratio was 25:1 (v/v). Extractions were carried out at 4° for 18 hr. The precipitated proteins were sedimented by centrifugation at 3000 *g* for 10 min. The supernatant was removed and the precipitate was dried under a stream of nitrogen [16]. VLDL and HDL apoproteins were solubilised in Tris–HCl 1 mM, 9 g/l NaCl buffer. LDL apoproteins were solubilised in urea 8 M, 9 g/l NaCl.

The protein content of each extract was determined by Lowry's method and purity was checked by electrophoresis [17].

Platelet antiaggregants. Ticlopidine and PCR 2362 (Fig. 1) were kindly given by Parcor. Their solutions were prepared by isotopic dilution of a constant amount of [^{3}H]ticlopidine hydrochloride (5 Ci/mmole, CEA) and [^{14}C]PCR 2362 methanesulfonate (10 mCi/mmole, CEA) with increasing amounts of the respective unlabelled drugs.

Experimental methods

Drug binding was studied by equilibrium dialysis. The experiments were carried out at 37°, pH = 7.4, for 6 hr under a constant stirring at 20 rpm (Dianorm apparatus). No significant binding to the dialysis tubing (Visking) and cell walls was observed. Ticlopidine and PCR 2362 were used over respective ranges of concentrations 3–660 μM (1–200 μg/ml) and 2.8–570 μM (1–200 μg/ml). At the end of each experiment, concentrations in each compartment were measured by liquid scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer 3320). Human serum pool had the following characteristics: total serum proteins 70 g/l, HSA 637 μM (44 g/l), α_1-AGP 17 μM (0.75 g/l), apoproteins B 1 g/l and apoproteins A 2 g/l. HSA (Sigma A-1887) and α_1-AGP (Behring) were used at 600 μM (40 g/l) and 20 μM (0.9 g/l). Using the following molecular weights: 7.5×10^6 for VLDL, 3.5×10^6 for LDL and 3×10^5 for HDL, concentrations of these different lipoproteins were 0.45 μM (3.4 g/l), 1.4 μM (5.0 g/l) and 6.7 μM (2.0 g/l). According to Scanu *et al.* [18] and Osborne *et al.* [19] apoprotein content of each lipoprotein is 10, 20 and 50% for VLDL, LDL and HDL, respectively.

Apo-VLDL, apo-LDL and apo-HDL concentrations used were 0.23, 0.20 and 1.00 g/l, respectively.

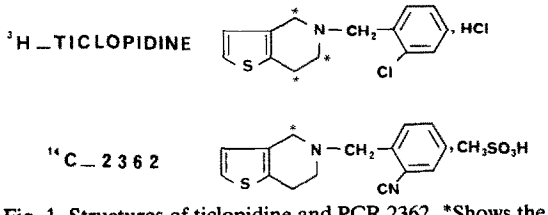

Fig. 1. Structures of ticlopidine and PCR 2362. *Shows the labelled positions, ^{3}H for ticlopidine and ^{14}C for PCR 2362.

Computation of binding parameters

Let the concentration of the protein be R and the total concentration of drug be T. At equilibrium, bound (B) and free (F) concentrations of drug were measured. Binding of these two drugs to HSA and lipoproteins was nonsaturable in the range of concentrations used so that B can be related to F by the following equation [20]:

$$B = (nK)\,RF \qquad (1)$$

where nK is the product of the number of binding sites, n, by the affinity constant K. Binding of these two drugs to α_1-AGP was saturable, so the binding equation was as follows [20]:

$$B = \frac{n\,KRF}{1 + KF} \qquad (2)$$

Apoproteins binding parameters were given as NK expressed in g/l of proteins because molecular weights of these fractions are unknown (NK is the product of the binding sites concentration by the affinity constant).

Finally, the product nK or/and NK represents the slope of the tangent from the origin. A curve B = f (F) was plotted and then all parameters n, K, nK and NK were estimated by means of the non-linear least squares method using Gauss–Newton algorithm [20].

RESULTS

Drugs binding to serum and HSA

The percentage of both drugs bound to either HSA or serum remained constant over the range of concentrations studied. However, binding was significantly higher in serum than in a pure HSA solution (Table 1). This difference was even more pro-

Table 1. Ticlopidine and PCR 2362 binding percentages and parameters to serum and HSA.

DRUGS	PROTEINS	BINDING (%)	nK (M^{-1})	NK
TICLOPIDINE	HSA	86.7 ± 1.5	9394 ± 329	5.64 ± 0.19
	Serum	97.8 ± 0.1		42.70 ± 0.60
PCR 2362	HSA	82.0 ± 1.2	7121 ± 65	4.27 ± 0.04
	Serum	93.5 ± 0.3		14.35 ± 0.21

[Ticlopidine], 3–660 μM; [PCR 2362], 2.8–570 μM; [HSA], 600 μM.

In serum, [HSA] = 637 μM and [α_1-AGP] = 17 μM.

nK is the product of the number of binding sites (n) by the corresponding affinity constant (K). NK, binding capacity, is the product of the binding sites concentration (N) by the affinity constant (K).

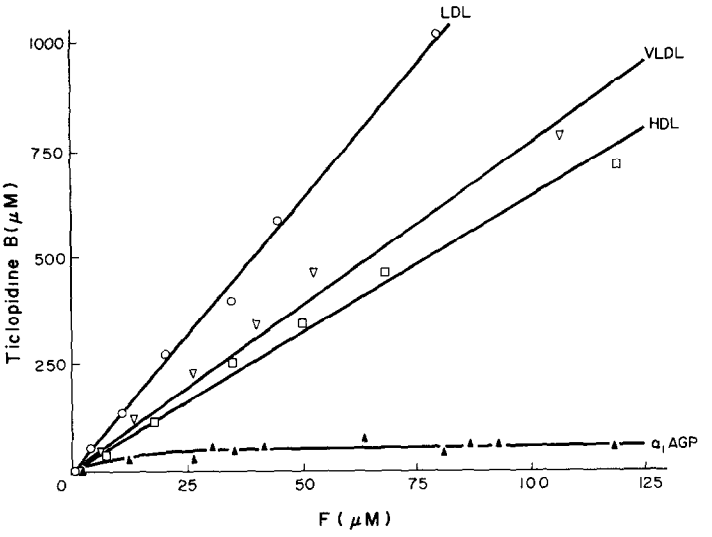

Fig. 2. Ticlopidine binding to α_1-AGP and lipoproteins. [Ticolopidine], 3–660 μM; [α_1-AGP], 20 μM; [VLDL], 0.45 μM; [LDL], 1.4 μM; [HDL], 6.7 μM.

nounced when binding capacities (*NK*) of serum and HSA were compared, suggesting that in serum, other proteins, in addition to HSA, are involved in the binding of these drugs. Under our experimental conditions, drug binding appeared to follow a non saturable process both to serum and to HSA.

Drugs binding to α_1-AGP

The two drugs are bound to α_1-AGP by a saturable process (Figs. 2 and 3). They have the same number of binding sites, respectively 3.04 ± 0.26 and 2.99 ± 0.23 for ticlopidine and PCR 2362 but different association constants, $88{,}800 \pm 22{,}000\ M^{-1}$ and $33{,}000 \pm 8500\ M^{-1}$.

Drugs binding to VLDL, LDL and HDL

The binding of ticlopidine and PCR 2362 to lipoproteins was non-saturable (Figs. 2 and 3). Ticlopidine binding to VLDL, LDL and HDL was quantatively important (>87%) (Table 2) and the binding capacities (*NK*) of the three proteins were higher than that of HSA (Table 1). In contrast, PCR 2362 was not so strongly bound to VLDL, LDL and HDL (≃ 65%) (Table 2) and the respective binding capacities were lower than that of HSA (Table 1). The apoprotein binding capacities (*NK*) for these two drugs were at least ten times lower than the native lipoproteins (Table 3).

Drugs binding to serum

Serum binding capacities are shown in Table 4. They do not differ from the sum of the five individual binding capacities. Thus binding to these different proteins can account for the observed total serum binding.

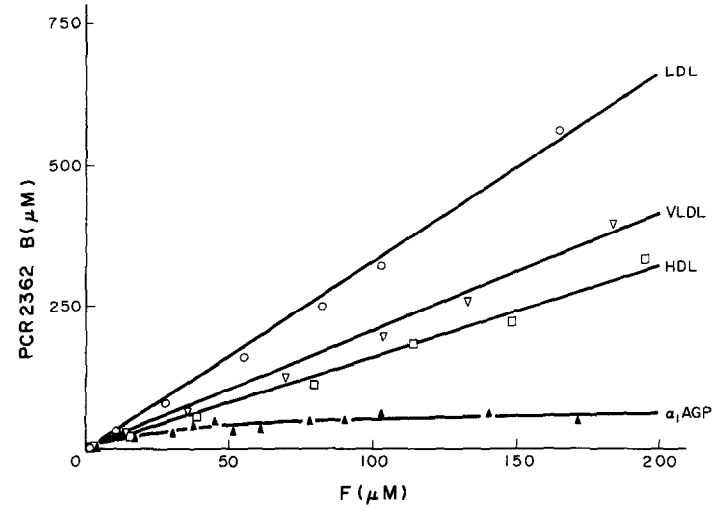

Fig. 3. PCR 2362 binding to α_1-AGP and lipoproteins. [PCR 2362], 2.8–570 μM; [α_1-AGP], 20 μM; [VLDL], 0.45 μM; [LDL], 1.4 μM; [HDL], 6.7 μM.

Table 2. Ticlopidine and PCR 2362 binding percentages and parameters to lipoproteins

	Lipoproteins	VLDL	LDL	HDL
Ticlopidine	Binding (%)	89.2 ± 1.1	92.8 ± 0.5	87.1 ± 1.1
	nK . 10^{-6}(M^{-1})	16.95 ± 0.28	8.89 + 0.51	1.01 ± 0.19
	NK	7.62 ± 0.13	12.67 ± 0.71	6.40 ± 1.20
PCR 2362	Binding (%)	64.7 ± 2.3	74.7 ± 1.4	58.3 ± 1.6
	nK . 10^{-6}(M^{-1})	4.54 ± 0.06	2.30 ± 0.07	0.25 ± 0.05
	NK	2.04 ± 0.03	3.30 ± 0.10	1.60 ± 0.32

Drug concentrations, see Table 1.
[VLDL], 0.45 μM (0.34 g/l of proteins); [LDL], 1.4 μM (1.00 g/l of proteins); [HDL], 6.7 μM (1.00 g/l of proteins).

Table 3. Ticlopidine and PCR 2362 binding capacities (*NK*) to lipoproteins and apoproteins

		Lipoproteins	Apoproteins
Ticlopidine	VLDL	22.58 ± 0.86	2.80 ± 0.15
	LDL	12.67 ± 0.71	0.43 ± 0.02
	HDL	6.40 ± 1.20	0.74 ± 0.03
PCR 2362	VLDL	6.06 ± 0.19	0.26 ± 0.01
	LDL	3.30 ± 0.07	0.09 ± 0.01
	HDL	1.60 ± 0.05	0.12 ± 0.01

NK are expressed in g/l of proteins. Binding capacities of native lipoproteins were calculated from *NK* of Table 2 and expressed in g/l of proteins, e.g. ticlopidine *NK* for VLDL 7.62 for 0.34 g/l of proteins becomes 22.58 for 1 g/l of proteins. Binding capacities of apoproteins were reset to 1 g/l of proteins.

Table 4. Ticlopidine and PCR 2362 calculated binding capacities (*NK*) in a normal serum

Proteins (μM)	HSA (600)	α_1AGP (20)	VLDL (0.16)	LDL (1)	HDL (13)	Serum (635)
Ticlopidine	5.6	5.3	2.7	8.9	13.0	35.5
PCR 2362	4.3	2.0	0.7	2.3	3.3	12.6

[HSA] and [α_1-AGP] are identical to concentrations used in experimental conditions. Lipoprotein physiological concentrations are different from those used in experimental conditions.

DISCUSSION

Drug binding to HSA

From the present results, it is clear that these two drugs are not only bound to HSA in serum. So the apparent discrepancy is explained by their high binding percentages in serum and their relatively low binding capacities to HSA. The same phenomenon has already been described for many basic drugs, including propranolol [11] and quinidine [21, 22]. It appears to be a general phenomenon for basic drugs. The other interesting feature of the interaction of ticlopidine and PCR 2362 with HSA, is that binding appears to be non saturable. This finding distinguishes these drugs from acidic ones which follow a saturable process that is characterized by a definite number of HSA binding sites with high association constants. The non saturable nature of basic drug binding to HSA probably involves a hydrophobic interaction between hydrophobic HSA areas and lipophilic regions in these molecules [23].

Drugs binding to α_1-AGP

The two drugs bind to α_1-AGP by a saturable process. As reported by Piafsky *et al.* [4], it is a general phenomenon for basic drugs. However, in this work, three α_1-AGP binding sites for ticlopidine and PCR 2362 have been observed instead of one for other drugs. In normal serum, i.e. serum proteins being in normal range, α_1-AGP binding capacity (*NK*) represents 15% of total calculated serum binding capacity. It can be predicted that in pathological states, where α_1-AGP concentration increases [4], the corresponding binding capacity may proportionally increase; it can be calculated that, when the α_1-AGP concentration doubles, its corresponding drug binding capacity may increase from 15 to 26% of the total serum binding capacity. This prediction may be verified by measuring binding in serum from patients suffering from inflammatory diseases.

Drugs binding to lipoproteins

For the two drugs, the decrease in *nK* (VLDL > LDL > HDL) appeared to correlate well with the respective lipid contents of these lipoproteins (90–80 and 50%) [18]. In addition the *nK* values were related to the partition coefficients of these drugs. Similar results have been obtained for propranolol [11] and quinidine [9]. Since, in addition binding followed a non saturable process, it would appear that these drugs are dissolved in the lipid portion of the lipoproteins. In normal serum, lipoprotein binding capacity represents 70% of the total (calculated) serum binding capacity of these drugs. It can also be predicted that in hyperlipidemia type IIb where the concentration of VLDL and LDL increase, the corresponding binding capacity would also increase from 70 to 77%. This small change is due to the large lipoproteins binding capacity for these drugs under physiological conditions. The binding capacities of isolated apolipoproteins are low and represent less than 10% of the lipoprotein binding capacities. Thus it seems likely that the protein fractions are not important for binding of these drugs. It should be emphasized, that, even though the apoprotein electrophoretic patterns were correct, the delipidation conditions are rather drastic and probably result in a tertiary structure that may differ from that in the native lipoprotein.

Drugs binding to serum

From these results, it is clear that these two drugs are bound to five main serum proteins, HSA, α_1-

AGP, VLDL, LDL and HDL. This distribution pattern of the serum bound drug has also been found for other basic drugs, e.g. quinidine [9], propranolol [11] and partly demonstrated for imipramine, nortriptyline and chlorpromazine [7]. However, the basic drug, erythromycin does not bind to lipoproteins but interacts with HSA and α_1-AGP via non saturable and saturable processes, respectively. This lack of lipoprotein binding is probably due to the hydrophilic nature of this molecule [24]. Thus it appears that this multiple serum binding may be a common feature for many basic drugs provided that they have a certain minimum lipid solubility which has yet to be determined.

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