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The blood binding of cefotiam and cyclohexanol, metabolites of the prodrug cefotiam hexetil, in-vitro

V. QUEROL-FERRER, R. ZINI, J.-P. TILLEMENT, *Laboratoire Hospitalo-Universitaire de Pharmacologie de l'Université Paris XII, 8 rue du Général Sarrail, F-94010 Créteil, France*

Abstract—The binding of cefotiam and cyclohexanol to human serum, isolated proteins and erythrocytes has been studied in-vitro by equilibrium dialysis. The two molecules are 50% bound to serum proteins and the free fraction for both compounds remained constant within the therapeutic concentration range. Human serum albumin (HSA) was exclusively responsible for the cefotiam binding (48%) with a saturable process characterized by one binding site ($n = 1{\cdot}00 \pm 0{\cdot}14$) with a very weak affinity ($K_a = 1457 \pm 352\ M^{-1}$). Like other cephalosporins, cefotiam showed no binding to α_1-acid glycoprotein, lipoproteins or γ-globulins. Cyclohexanol is mainly bound to HSA with a weak affinity ($K_a \approx 1\,800\ M^{-1}$) but lipoproteins and α_1-acid glycoprotein bind about 30% of bound cyclohexanol in serum. Interactions with free fatty acids (FFA) or bilirubin were studied at physiopathological concentrations. HSA-bound cefotiam was displaced by FFA (1260 μM) and bilirubin (330 μM), whereas the cyclohexanol binding was inhibited only by FFA. The cefotiam binding site seems to be close to the warfarin site (site I) whereas cyclohexanol probably shares the diazepam site (site II) on HSA. There is no mutual inhibition of binding between cefotiam and cyclohexanol at therapeutic levels. The binding of both compounds to erythrocytes is low and restricted when measured in the presence of plasma.

Cefotiam hexetil (SCE 2174; Takeda-Cassenne), a new oral third-generation cephalosporin, is a prodrug of cefotiam (Pansporine) and has no anti-bacterial properties. After oral administration, cefotiam hexetil is hydrolysed in the gut and absorbed as cefotiam. The main metabolite found in blood is cyclohexanol.

The purpose of this study was to determine to what extent cefotiam and cyclohexanol are bound in serum and to identify the proteins responsible for binding. Studies were first performed in normal human serum and then the binding of both molecules was studied on the main isolated proteins. In addition, possible binding to red blood cells and interactions with endogenous or exogenous compounds were checked. These experiments were carried out by equilibrium dialysis.

Materials and methods

Human serum. A pool of human serum was obtained after collection of blood samples from healthy volunteers. The human serum had the following characteristics: total proteins = 75 g L^{-1}, human serum albumin (HSA) = 770 μM, α_1-acid glycoprotein (AAG) = 11 μM. The concentration of free fatty acids (FFA) was 336 μM and the molar ratio FFA/HSA was 0·44. Bilirubin was present at a concentration of 8 μM. The serum pool was stored at −30°C before use.

Human serum albumin. HSA (Sigma A-1887, purity 99%, FFA/HSA = 0·04) was dissolved in phosphate buffer (KH_2PO_4, Na_2HPO_4), 67 mM, pH 7·4, at a concentration of either 300 or 770 μM.

α_1-Acid glycoprotein (AAG). AAG (Behring electrophoresis, purity 99%) was dissolved in 67 mM phosphate buffer, pH 7·4, at a concentration of 25 μM.

Correspondence: J. P. Tillement, Laboratoire Hospitalo-Universitaire de Pharmacologie de l'Université Paris XII, 8 rue du Général Sarrail, F-94010 Créteil, France.

Lipoproteins. Very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins obtained by ultracentrifugation (Beckman L5-50B, rotor 50 Ti) of pooled normolipidaemic human serum by Nelson's method (Nelson 1980) as modified by Glasson et al (1982). The concentrations measured by the method of Lowry et al (1951) were 0·1, 1 and 10 μM for VLDL, LDL and HDL, respectively.

γ-Globulins. IgG (Sigma HGII, purity 99%) was dissolved in phosphate buffer at a concentration of 100 μM.

Red blood cells (RBC). RBCs were washed three times in 0·9% NaCl (saline) and adjusted to a haematocrit of 0·45 in an isotonic saline glucose buffer composed as follows (mM): glucose 5·0, KH_2PO_4 5·0, $Na_2HPO_4 \cdot 12H_2O$ 20·0, $CaCl_2$ 2·5, $MgSO_4$ 1·0, NaCl 100·0, pH 7·4 at 37°C. In addition the same haematocrit was reconstituted in the serum pool described above and the washed RBCs. [^{14}C]Cefotiam or [^{14}C]cyclohexanol and RBCs were incubated at 37°C for 15 min, then centrifuged at 1000 *g* at 4°C for 15 min. Drug concentrations were determined in RBCs suspension, in plasma and in buffer.

Drugs. Cefotiam. ^{14}C-Labelled and unlabelled cefotiam were supplied by Cassenne-Takeda Laboratories (Japan). The specific activity of [^{14}C]cefotiam was 24·3 Ci mol^{-1} (radiochemical purity > 96·7%). Cefotiam was dissolved in methanol to obtain a 10 mM solution. Further dilutions were then prepared in 67 mM phosphate buffer, pH 7·4.

Cyclohexanol. [^{14}C]Cyclohexanol (Sp. act.: 57 Ci mol^{-1}, radiochemical purity > 96%) was from Amersham (UK). A 1 mM solution was made in methanol and the different dilutions in 67 mM phosphate buffer, pH 7·4. Unlabelled cyclohexanol was from Merck (Germany). The other compounds were as follows: warfarin (Merrell-Toraude, France), diazepam (Roche, Switzerland), binedaline (Cassenne), bilirubin (Merck, Germany). Diazepam was added into the protein compartment in a volume of 4 μL to keep the alcohol content below 5%.

Equilibrium dialysis. The binding of [^{14}C]cefotiam and [^{14}C]cyclohexanol to various isolated proteins and to serum was studied by equilibrium dialysis, with a Dianorm apparatus. All experiments were carried out for 3 h at 37°C with Spectrapor membranes. At the end of each experiment, concentrations in both compartments were measured by liquid scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer 460 CD).

Calculations. Free (F) and bound (B) concentrations of [^{14}C]cefotiam and [^{14}C]cyclohexanol were calculated. The percentage bound (f_b) was determined from the concentrations obtained at equilibrium:

$$f_b = \frac{B}{B + F} \times 100 \qquad (1)$$

The data obtained at equilibrium (B and F) were fitted according to the equation:

$$B = \sum_{j=1}^{m} \frac{N_j K_{aj} F}{1 + K_{aj} F} = \sum_{j=1}^{m} \frac{n_j R K_{aj} F}{1 + K_{aj} F} \quad (2)$$

where N_j, n_j, K_{aj} denote respectively the molar binding site concentration of the j class, the number of binding sites and the apparent association constant of the specific protein; R denotes the concentration of the protein. The parameters n and K_a were calculated by a non-linear least-squares method using a Gauss-Newton algorithm (Zini et al 1979). The binding percentages and estimated parameters were expressed as the mean ± s.d. of 3–5 determinations. Simulation of drug binding in plasma or blood was calculated at non-saturating concentrations.

The [^{14}C]cefotiam and [^{14}C]cyclohexanol concentrations in the RBC were obtained by:

$$C_{RBC} = \frac{C_{blood} - C_{plasma}(1 - H)}{H} \quad (3)$$

where H is the haematocrit value.

The fraction of cefotiam or cyclohexanol in RBC (f_{RBC}) is calculated as follows:

$$f_{RBC} = \frac{C_{RBC}}{T} \times H \quad (4)$$

where T is the total concentration of the drug.

When a passive diffusion occurs, the fraction of cefotiam or cyclohexanol in RBC (f'_{RBC}) then becomes:

$$f'_{RBC} = \frac{H}{1 + \frac{f_b}{f_u}(1 - H)} \quad (5)$$

where f_u and f_b represent respectively the free and bound fractions of the drug in plasma.

Results

Cefotiam and cyclohexanol binding to human serum. The percentage bound of both drugs remained constant from 1 to 10 μM. The mean values were 44·5 ± 2·1% for cefotiam and 53·1 ± 0·8% for cyclohexanol (Table 1). These parameters led to the calculation of binding coefficients (nK_a) of 0·805 ± 0·067 and 1·134 ± 0·04, respectively.

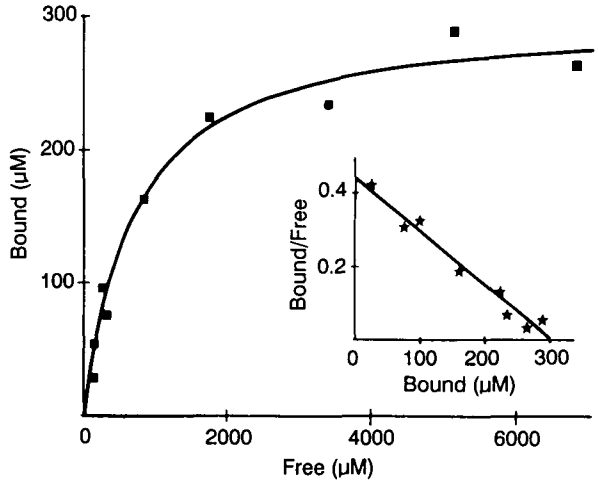

FIG. 1. Binding of cefotiam to HSA 300 μM. B and F are respectively the concentrations of bound and free cefotiam. Each point represents the mean ± s.d. of three determinations. The insert is the Scatchard plot.

Cefotiam and cyclohexanol binding to HSA. Effect of FFA and bilirubin. The percentage of cefotiam bound to 770 μM HSA remained constant between 1 and 10 μM (47·22 ± 1·26%) and was slightly higher than that measured in serum.

With 300 μM HSA and at higher concentrations of cefotiam (1–7500 μM) the results showed one saturable binding site (n = 1·00 ± 0·14) with a weak affinity (K_a = 1457 ± 352 M^{-1}) (Fig. 1).

For cyclohexanol concentrations up to 10 μM, the bound fraction with 770 μM HSA was constant (58·79 ± 1·69%) but significantly higher than in serum. To avoid the presence of high alcoholic concentrations that could denature the albumin, we did not use a wide range of cyclohexanol concentrations. Nevertheless an indirect method with a fixed concentration of cyclohexanol (1 μM) and several HSA concentrations (20–500 μM) allowed an estimation of the binding coefficient of cyclohexanol on HSA as K_a = 1800 M^{-1}.

The difference of binding observed between serum and HSA led us to check the role of FFAs as displacing agents. The

Table 1. Cefotiam and cyclohexanol binding to human serum and isolated proteins. Each percentage represents the mean ± s.d. of 3 determinations. Statistical differences were calculated using Student's *t*-test.

	Binding (%)					
	Cefotiam (μM)			Cyclohexanol (μM)		
	1	5	10	1	5	10
Proteins (μM)						
Serum	45·2 ± 2·3	42·2 ± 3·2	46·2 ± 3·1	52·2 ± 2·8	53·8 ± 1·6	53·4 ± 0·7
HSA (770)	47·4 ± 1·5	47·3 ± 1·4	48·6 ± 1·4	58·7 ± 1·8**	56·7 ± 2·7*	59·5 ± 1·4**
HSA (770) + FFA (1260)	41·0 ± 0·7##	—	43·1 ± 1·0##	51·4 ± 1·9**	—	51·9 ± 0·8**
Serum + bilirubin (330)	39·8 ± 1·3**	—	39·7 ± 2·1**	52·7 ± 1·1	—	53·0 ± 1·2
AAG (25)	2·6 ± 2·4	—	—	22·3 ± 1·6	—	—
VLDL (0·1)	1·7 ± 1·0	—	—	10·9 ± 0·1	—	—
LDL (1)	3·1 ± 2·3	—	—	26·5 ± 1·2	—	—
HDL (10)	3·6 ± 1·1	—	—	30·0 ± 1·3	—	—
γ-Globulins	4·1 ± 1·6	—	—	1·9 ± 0·4	—	—

$P < 0·01$ vs 770 μM HSA; * $P < 0·05$ vs serum; ** $P < 0·01$ vs serum.

Table 2. Cefotiam and cyclohexanol binding to HSA (300 μM) in the presence of warfarin or diazepam. Each percentage is the mean ± s.d. of 5 determinations. Statistical differences were calculated using Student's *t*-test.

	Binding (%)	
	Cefotiam (5 μM)	Cyclohexanol (5 μM)
Drugs		
None	25·9 ± 1·6	53·8 ± 0·8
Warfarin (1 mM)	10·0 ± 0·5**	52·4 ± 0·4
Diazepam (2 mM)	20·3 ± 1·3**	38·2 ± 1·5**

**$P < 0.01$ compared with the respective binding of cefotiam and cyclohexanol alone.

presence of FFA (palmitic acid, 1260 μM) in HSA induced a significant decrease in the binding of the two drugs. This inhibitory effect was more pronounced for cyclohexanol than for cefotiam (Table 1). When bilirubin (300 μM) was added to serum the cefotiam binding decreased compared with that observed in pooled normal serum whereas cyclohexanol binding was not affected (Table 1).

Cefotiam and cyclohexanol binding to AAG, lipoproteins and γ-globulins. Cefotiam displayed no binding to either AAG (25 μM), lipoproteins or γ-globulins at physiological concentrations. On the other hand, cyclohexanol showed moderate binding to 25 μM AAG (22%) and to native lipoproteins at physiological concentrations (HDL: 30%, LDL: 26%, VLDL: 11%) but not to γ-globulins (Table 1).

Localization of cefotiam and cyclohexanol binding sites on HSA and AAG. [^{14}C]Cefotiam (5 μM) binding to HSA (300 μM) was decreased when saturating concentrations of warfarin (1 mM) and diazepam (1 mM) were used (Table 2). The greatest inhibition occurred with warfarin. On the other hand, [^{14}C]cyclohexanol was displaced by diazepam but not at all by warfarin. Binedaline was used as a probe to determine the cyclohexanol site on AAG. [^{14}C]Cyclohexanol (5 μM) binding to AAG (25 μM) was almost completely inhibited by binedaline (100 μM), the percentage of binding falling from 28·4 to 4·6%.

Cefotiam and cyclohexanol binding to RBCs. Over a range of concentrations from 5 to 200 μM the binding of cefotiam and cyclohexanol to RBC (f_{RBC}) was constant and non-saturable (Table 3). The erythrocyte cefotiam and cyclohexanol bound fractions in the presence of phosphate buffer were respectively twice and four times higher than in the presence of plasma,

Table 3. Binding (%) of cefotiam and cyclohexanol to erythrocytes. $f_{RBC}B$ = fraction bound to RBCs in the presence of buffer, $f_{RBC}P$ = fraction bound to RBCs in presence of plasma, f'_{RBC} = fraction in RBCs if no binding occurs to the cells (passive diffusion only).

	$f_{RBC}B$	$f_{RBC}P$	f'_{RBC}	$f_{RBC}B/f'_{RBC}$
Cefotiam (μM)				
5	22·8 ± 3·4	11·5 ± 9·2	31	0·4
10	16·6 ± 2·8	12·4 ± 7·9	31	0·4
20	25·1 ± 7·4	15·0 ± 10·1	31	0·5
100	35·7 ± 3·0	16·9 ± 3·4	31	0·5
200	36·3 ± 4·2	12·7 ± 6·1	31	0·4
Cyclohexanol (μM)				
5	39·5 ± 15·6	8·5 ± 1·6	28	0·3
10	25·8 ± 10·8	4·6 ± 2·0	28	0·2
20	40·5 ± 5·5	12·6 ± 2·3	28	0·4
100	44·0 ± 11·2	12·2 ± 4·3	28	0·4
200	52·0 ± 9·5	13·3 ± 1·1	28	0·5

indicating the importance of the binding to plasma proteins despite their low affinities. Moreover the ratio f_{RBC}/f'_{RBC} was lower than unity, showing that the diffusion into RBC is very low (Table 3).

Discussion

The results show that cefotiam is weakly bound to plasma proteins (45%) and that its binding remains constant within the range of therapeutic concentrations (1 − 10 μM). Using the estimated binding parameters of cefotiam to the different blood fractions, we simulated its distribution in blood at therapeutic levels: cefotiam is 39·5% bound to HSA, other proteins representing 6% of total binding. The participation of RBCs in total blood binding is low and represents about 10% of the whole binding. Cefotiam binding to HSA is higher than that obtained with human serum or with HSA + FFA (Table 1). This phenomenon can be explained by the ability of FFA to displace cefotiam from its HSA binding site. As reported by Tillement et al (1984), HSA would possess five binding sites with only two for endogenous ligands (FFA and bilirubin). Binding of FFA to their own sites leads to a structural change of HSA and thus to a decrease of the cefotiam binding (Birkett & Wanwimolruk 1985). Bilirubin has an inhibitory effect on the binding of cefotiam to serum proteins (Table 1). Bilirubin is known to have one high affinity binding site and one or two sites with a low affinity on HSA (Brodersen et al 1984). In our work, the bilirubin/HSA ratio was 0·5. Thus, HSA-bilirubin binding is non-saturable, bilirubin only occupying its first site. The decreased cefotiam binding could be explained by an allosteric effect induced by the binding of bilirubin to its high affinity site.

Cefotiam binding to 300 μM HSA is saturable with one site of low affinity ($\approx 1500\ M^{-1}$). The profile of its binding is close to that of many cephalosporins which bind only to HSA, e.g. cefuroxime, cefotaxime or cefazolin (Watanabe et al 1980; Barrière et al 1982; Nakagawa et al 1982; Decroix et al 1988). Cefotiam shares or is close to the warfarin binding site (site I) as shown by inhibition studies (Table 2) (Maruyama et al 1986). However, diazepam is able partly to displace cefotiam (Table 2). Two hypotheses may be proposed: either diazepam binding to its own site (site II) leads to a conformational change in HSA entailing a decrease of cefotiam binding to site I or the cefotiam binding site is very close to site II; the cefotiam site could occupy a spanning area overlapping sites I and II.

Cyclohexanol, the main inactive metabolite found after cefotiam hexetil absorption, is 52% bound in serum. When studied on isolated proteins, its binding is mainly to HSA but with a small contribution from AAG and especially of lipoproteins (Table 1). The simulation of the blood distribution of cyclohexanol shows that HSA, AAG, lipoproteins and RBCs respectively bind 31, 3·5, 25 and 12% of the drug. Surprisingly, the binding percentage measured in serum is lower than that to HSA alone (Table 1). This discrepancy can be explained by the displacing effect of FFA on cyclohexanol binding (Table 1). Since cyclohexanol binding to native isolated lipoproteins decreases from HDL to VLDL, cyclohexanol probably binds to the non-lipophilic moiety of the macromolecules. Cyclohexanol bound to AAG can be displaced by binedaline, a molecule exhibiting a very high affinity ($K_a = 2 \cdot 10^6\ M^{-1}$) for AAG (Morin et al 1985). Thus, cyclohexanol shares the same binding site on the α_1-acid glycoprotein as binedaline, tricyclic antidepressants (Piafsky & Borga 1977), neuroleptics (Muller & Stillbauer 1983) and β-blockers (Borga et al 1977). On HSA, cyclohexanol selectively binds to the diazepam site (Table 2) with a weak affinity ($\approx 1800\ M^{-1}$). This binding to HSA does not seem to be inhibited by high concentrations of warfarin (Table 2) but the hypothesis of an intermediate site, as with cefotiam, cannot be

ruled out. Such interactions are not likely to happen in therapeutics because cyclohexanol, as well as cefotiam, has subsaturating plasma levels. On the other hand, neither cyclohexanol nor cefotiam could displace drugs bound to site I or II because of their weak affinities; saturation of HSA binding sites could only occur with cefotiam or cyclohexanol concentrations above 1 mM greatly in excess of therapeutic plasma levels.

The binding of cyclohexanol and cefotiam to RBCs follows a non-saturable process within the therapeutic range. The erythrocyte bound fraction is higher in phosphate buffer than in plasma (Table 3) indicating a retention of both compounds by the plasma proteins. Intra-erythrocyte fraction of the two drugs is low (Table 3). This would tend to indicate that they cannot easily penetrate RBC membranes by passive diffusion.

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Receptor binding at two different temperatures to discriminate agonist and antagonist behaviour of adenosine A_1 receptor ligands in rat brain

PIER ANDREA BOREA, KATIA VARANI, VALERIA MALAGUTI, GASTONE GILLI*, *Istituto di Farmacologia and *Dipartimento di Chimica Universita di Ferrara, 44100 Ferrara, Italy*

Abstract—The inhibitory binding constants, K_i, at the adenosine A_1 receptor in rat brain have been measured at 0 and 25°C for 25 typical ligands. The K_i ratios at the two temperatures are greater and smaller than unity for adenosine agonists and xanthine antagonists, respectively. These results suggest that two-temperature measurements of in-vitro K_i constants represent a simple method of discriminating between in-vivo agonistic and antagonistic behaviour of A_1 adenosine receptor ligands.

In general, receptor binding measurements give accurate information on drug receptor affinities but little or none about the nature of the effect which therefore has to be determined by independent in-vitro and in-vivo experiments. However, drug design and testing would be expedited by a method able to discriminate between agonist and antagonist properties by simple affinity constant measurements. This has been proved possible in some cases by adding specific substances able to modulate in different ways the binding of drugs which produce different effects. For instance, it is known that prior incubation of brain membranes with γ-aminobutyric acid (GABA) modulates the binding of benzodiazepine receptor ligands in such a way that the ligand agonistic, antagonistic and inverse agonistic behaviour can be discriminated (Möhler & Richards 1981). Moreover, it is generally recognized that the presence of certain ions, e.g. Na^+ or Mg^{2+}, can modulate in different ways the binding of agonists and antagonists in several receptor systems (Creese 1985). As far as the adenosine A_1 receptor is concerned, it has been reported (Goodman et al 1982) that guanine nucleotides and divalent cations affect agonist and antagonist binding differently; in particular, GTP (guanosine-5′-triphosphate) is currently employed for its property of reducing agonist but not antagonist affinity at this receptor.

A different way of modulating the recognition process of

Correspondence: P. A. Borea, Istituto di Farmacologia, via Fossato di Mortara 64/B, 44100 Ferrara, Italy.