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Comparative binding of etretinate and acitretin to plasma proteins and erythrocytes

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Abstract—The interactions of etretinate and its main metabolite acitretin with human plasma proteins have been investigated *in vitro* by an erythrocyte partitioning technique that allows a quantitative estimation of the plasma and erythrocyte binding. Etretinate was extensively lipoprotein-bound (75% of plasma etretinate), with a binding constant for its main low density lipoprotein carrier of $40 \times 10^6\ M^{-1}$, accounting for 48% of the total plasma-bound drug. Acitretin was mainly albumin-bound (91% of plasma acitretin), with a binding constant of $0.7 \times 10^6\ M^{-1}$. The total plasma binding of both drugs was >99% and, in blood, the fractions associated with erythrocytes were 14.5 and 8.1% of the total amount for etretinate and acitretin, respectively.

It is generally accepted that free drug concentration is more closely related to the pharmacological or toxicological response than total drug concentration in plasma. This applies particularly to drugs with a high degree of plasma protein binding such as etretinate and acitretin, synthetic retinoids used in the treatment of skin diseases [1]. These drugs exhibit high lipophilicity and are too hydrophobic to be dissolved in water. Thus, protein binding determination using a classical method such as equilibrium dialysis or ultrafiltration could provide unreliable results because of a high degree of non-specific adsorption on cells or membranes. Moreover, the equilibrium time for this kind of drug is generally longer than 3 hr with conventional material, which can result in drug degradation. These drugs have been reported to bind very strongly to plasma proteins [2, 3]. However, results for retinoid binding to plasma proteins have been obtained using methods that separate the bound fractions of ligand and thus could involve some change in the multiple equilibria between ligand bound and free forms.

The aim of the present study was to investigate the protein and erythrocyte binding of etretinate and acitretin by an alternative and fast method that measures the partitioning of the drug between isolated proteins or plasma solutions and erythrocytes. This method provides a biological dialysis system, in which protein solutions and erythrocytes are two compartments separated by the erythrocyte membrane. It has already been used to study the plasma protein binding of cyclosporin A [4] and retelliptine [5]. The results of the present study demonstrate the usefulness of this method to investigate retinoid protein binding.

Materials and Methods

Materials. All chemicals were of analytical grade. [^{3}H]-Etretinate (230 Ci/mol) and [^{14}C]acitretin (48.9 Ci/mol) were kindly supplied by F. Hoffmann-LaRoche (Basel, Switzerland). The radiochemical purity was greater than 98.5% as checked by TLC. Blood was withdrawn from the antecubital vein of a healthy volunteer (male, 37 years), free of drugs, and collected in plastic tubes containing EDTA (15 mg/10 mL of blood). Plasma was separated from erythrocytes by centrifugation at 3000 rpm for 3 min. Erythrocytes were washed three times with NaCl 9 g/L before use. Human proteins used were serum albumin (HSA,* fatty acid-free, Sigma A-1887), α_1-acid glycoprotein (AAG, Behring) and γ-globulins (GG, Sigma Cohn's fraction II) and lipoproteins: very low density lipoprotein (VLDL) fraction (Sigma L-2264), low density lipoprotein (LDL) fraction (Sigma L-2139) and high density lipoprotein (HDL) fraction (Sigma L-2014).

Methods. The erythrocyte partitioning method assumes that the free concentration of drug (C_u) in plasma is in equilibrium with that in erythrocytes. A 10 μL aliquot of radiolabelled ligand dissolved in ethanol was added to 2 mL of an erythrocyte suspension (20% hematocrit) in protein-buffered solution (pH 7.4, 120 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM Tris). The samples in capped glass vials were incubated (37°, 20 min) with gentle orbital shaking in a Brunswick water bath. Duplicate 25 μL aliquots were taken for counting the whole suspension (*WS*). After centrifugation (2000 *g*, 15 min, in a preheated Z2320K Centrifuge, BHG, Gosheim, Germany) at 37°, duplicate 25 μL aliquots of supernatant were counted (*P*). The ratio of drug concentration in erythrocytes and protein solution (C_E/C_P) was calculated from the following equation:

$$\frac{C_E}{C_P} = \frac{(WS/P) - (1 - H)}{H} \quad (1)$$

where H denotes the hematocrit. As we used trace concentrations of etretinate or acitretin (8–15 nM or 1–2 μM, respectively), we may assume that the C_P/C_E ratio is linearly related to the protein concentration (P_t) in the solution (see Refs 4 and 5 for details):

$$\frac{C_P}{C_E} = \frac{1 + NK_A \times P_t}{1 + K_E} \quad (2)$$

where NK_A is the total binding constant for ligand interaction with a protein (product of N, number of binding sites, by K_A, corresponding association constant), and K_E is the binding coefficient for ligand interaction with erythrocytes (dimensionless, ratio of erythrocyte-bound to free ligand concentration). Accordingly, the C_P/C_E ratios were measured at a series of protein concentrations (P_t). These data were ascribed to Eqn 2 and analysed by an iterative non-linear regression program using the least-squares criterion (MicroPharm, INSERM, 1990). In the case of plasma binding data, the terms K_P (bound-to-free concentration ratio or binding ratio, dimensionless) and f (fraction of plasma in buffer) are substituted for NK_A and P_t.

Concentrations of [^{3}H]etretinate or [^{14}C]acitretin in protein solution and erythrocyte suspension were determined in duplicate in a Packard liquid scintillation counter

* Abbreviations: HSA, human serum albumin; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; AAG, α_1-acid glycoprotein; GG, γ-globulins.

(Tri-Carb 460 CD). Twenty-five microlitres of solution or erythrocyte suspension were added to 3 mL of liquid scintillation solution (Pico-Fluor™). Prior to counting, the erythrocyte suspension was bleached by the following procedure: 25 μL of erythrocyte suspension were mixed with 200 μL sodium hypochlorite by Vortex agitation for 10 sec. The mixture was revortexed 10 min later and 3 mL of liquid scintillation solution were added to the resulting sample for counting. Control (non-radioactive) erythrocytes were added to the protein solutions and processed as above, to get comparable counting conditions.

Results and Discussion

The time to reach equilibrium for ligand distribution between erythrocytes and protein solution was measured by monitoring the ratio of erythrocyte-to-plasma concentration. Equilibrium was attained in less than 4 min. The time of incubation for subsequent experiments was then fixed at 20 min.

Representative data for etretinate binding to LDL are depicted in Fig. 1. Erythrocyte partitioning data obtained with all the proteins studied fit the partitioning model satisfactorily. Etretinate bound significantly to lipoproteins with binding constants that are roughly proportional to the lipid fraction of each lipoprotein (VLDL > LDL > HDL), then to HSA, whereas the binding to AAG and GG was insignificant. By contrast, acitretin was extensively bound to HSA, with similar association constant values observed for the binding of some polyene free fatty acids to HSA [6], and to a lesser extent to lipoproteins (Table 1).

Theoretical calculations (see Table 1) showed that lipoproteins, particularly the LDL fraction, were the main carriers in the blood for etretinate (76% of plasma etretinate), whereas HSA was mainly responsible for the plasma binding of acitretin (91% of plasma acitretin). HSA, because of its high plasma concentration, could also bind etretinate with a moderate affinity and this contributed significantly to the total plasma binding of etretinate. This roughly corroborates previous studies on etretinate and acitretin distribution among plasma proteins. Vahlquist *et al.* [2] showed by gel chromatography of plasma that lipoproteins and globulins contributed to 85 and 15% of etretinate plasma binding, respectively. In the study of Carillet *et al.* [3], etretinate was found to be distributed in almost equal amounts in HSA, HDL, LDL and VLDL, and acitretin was mostly distributed in HDL and in non-lipoprotein fractions. However, this was observed on sera mixed with high amounts of KBr and fractionated at 15° by ultracentrifugation, so the equilibrium observed is likely to be somewhat different from that under physiological conditions.

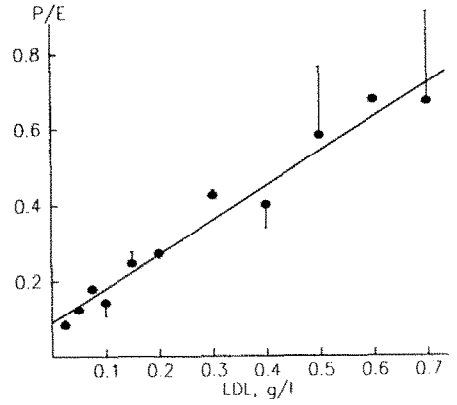

Fig. 1. Partitioning of etretinate between lipoproteins, LDL fraction, and erythrocytes at 37°. Each point is the mean ± SD of two determinations. The LDL solution containing a trace amount of [^{3}H]etretinate was incubated with erythrocytes (20% hematocrit). At equilibrium, the protein phase-to-erythrocyte concentration ratio (P/E) of etretinate was determined and is plotted vs protein concentration. Line is fitted according to Eqn 2.

Erythrocyte partitioning data obtained from erythrocytes suspended in a series of plasma dilutions in buffer (0.4–30%) served to determine the plasma binding ratio or binding coefficients of etretinate ($K_P = 135 \pm 40$) and acitretin ($K_P = 240 \pm 73$) and indicated that they were more than 99% and more than 99.5% plasma-bound. Erythrocyte binding coefficients were obtained from erythrocyte partitioning of the ligands with isolated plasma proteins and with diluted plasma (average K_E values: etretinate 21.0 ± 3.3, acitretin 31.6 ± 8.1).

Extensive tissue binding of etretinate can be deduced from its high distribution volume, V_{ss}, of 40 L/kg, which is in agreement with the extreme lipophilic nature of etretinate, whereas V_{ss} of acitretin is only 3.5 L/kg reflecting lower tissue binding and lipophilicity [6]. Etretinate tissue binding is likely to be favored by delivery via LDL endocytosis. It has been shown recently that retinyl ester could be taken up via the LDL receptor of the human promyelocytic cell line HL-60 [7] and that LDL might be used as carrier targeting lipophilic drugs to leukemic cells or to cells exhibiting elevated receptor-mediated uptake of LDL [8]. Accordingly, retinoic acid derivatives should be

Table 1. Binding parameters (NK_A) to isolated plasma proteins and plasma distribution of etretinate and acitretin

Protein	Binding constant, NK_A (mM^{-1})		% Bound in plasma	
	Etretinate	Acitretin	Etretinate	Acitretin
Albumin	10.5 ± 1.6*	668 ± 224	7.6	90.7
AAG	NS	257 ± 46	NS	0.6
GG	NS	97 ± 26	NS	3.7
HDL	831 ± 261	384 ± 42	12.4	2.3
LDL	39,600 ± 8130	4500 ± 1800	47.5	2.2
VLDL	156,500 ± 60,500	500 ± 200	15.6	NS

* Values are means ± SD.

NK_A, product of the number of sites by the association constant. NS, not significant.

The following molecular masses were used for the calculations: serum albumin 66, AAG 40, GG 180, HDL 3×10^2, LDL 3×10^3, VLDL 10^4 kDa. Plasma distribution of etretinate and acitretin is calculated assuming physiological concentrations of binding proteins: albumin 600, AAG 10, GG 80, HDL 11, LDL 1, VLDL 0.1 μM.

esterified when the therapeutic target tissue displays high LDL receptor endocytosis activity.

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