Biochemical Pharmacology, Vol. 37, No. 15, pp. 2963–2966, 1988.
Printed in Great Britain.

0006–2952/88 $3.00 + 0.00

BINDING OF INDAPAMIDE TO SERUM PROTEINS AND ERYTHROCYTES

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(*Received* 20 *August* 1987; *accepted* 22 *February* 1988)

Abstract—The binding of indapamide to isolated serum proteins and erythrocytes was studied in order to understand its blood distribution. In serum, indapamide was mainly bound to α_1-acid glycoprotein with a high affinity ($K = 73.4$/mM), and to albumin and lipoproteins. Indapamide was bound to erythrocytes via a saturable process with a high affinity ($K = 385$/mM and $N = 57$ μM for an hematocrit value of 0.48), and erythrocytes were the main binding component in blood (more than 80% of indapamide was associated to erythrocytes in blood). The binding to serum proteins affected indapamide distribution in blood, and α_1-acid glycoprotein was shown to be the more effective protein in decreasing the amount of indapamide associated to erythrocytes.

Indapamide (Fig. 1) is an oral antihypertensive agent with diuretic properties [1]. This drug is a lipophilic, acidic molecule ($pK_a = 8.3$), and at physiological pH (7.4), the drug is predominantly in an unionized state. By contrast with other acidic drugs, indapamide is mainly bound in blood to erythrocytes (90%) and exhibits a relatively low degree of serum binding (79%) [2].

In order to better understand indapamide distribution in blood, our objectives were to determine the serum binding and blood-to-plasma distribution of indapamide and to characterize its binding to isolated human serum proteins and erythrocytes.

MATERIAL AND METHODS

Drugs. Radiolabeled indapamide ([^{14}C]-S1520, 21.535 Ci/mol) was provided by the Institut de Recherches Internationales Servier, Neuilly, France. Stock solution of [^{14}C]-indapamide was prepared in ethanol. Radiochemical purity of the [^{14}C]-indapamide stock solution, as well as the stability of [^{14}C]-indapamide in the dialysate and protein solution after equilibrium dialysis was assessed by thin-layer chromatography on silica plates with the following development system: dichloromethane/acetone (80:20, v:v). More than 98% of the stock solution was pure, and no significant change in radiochemical purity was observed in either the dialysate or dialysed protein solution after equilibrium dialysis.

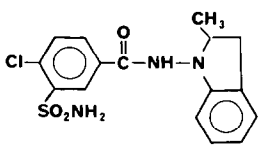

Fig. 1. Chemical structure of indapamide.

Blood fractions. All the proteins we used were of human origin. They included human serum albumin (HSA, Sigma A1887), α_1-acid glycoprotein (AAG, Behring), and serum lipoproteins, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) isolated by sequential ultracentrifugal flotation of serum at increasing density according to Glasson *et al.* [3]. The isolated lipoprotein fractions were diluted to the desired concentration with phosphate buffer and used without further modification in the equilibrium dialysis experiments. Erythrocytes and platelets were prepared from fresh blood samples, and AAG and HSA in serum were measured as reported earlier [4].

Binding experiments. Equilibrium dialysis, measurement of radioactivity and calculation of the binding parameters, i.e. n, number of binding sites, or N, concentration of binding sites ($N = n \cdot$ protein molar concentration), and K, association constant, were conducted as previously described in detail [4]. Equilibrium was achieved within 2 hr between the chambers containing plasma or protein solutions and 0.067 M Sörensen's buffer (pH 7.4) at 37°, without apparent accumulation of fluid on the protein side of the dialysis chamber. The drug did not bind to the apparatus. Indapamide was used at concentrations of 10–160 μM. Each sample was dialysed in duplicate.

Binding of indapamide to erythrocytes. Washed erythrocytes (see ref. [4]) were resuspended in normal (from a healthy subject) or pathological sera (from two patients with cancer disease) or in phosphate buffer saline (Sörensen's buffer plus 0.15 M

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NaCl, PBS) containing variable amounts of HSA or AAG. Known amounts of [^{14}C]-indapamide were added to test tubes containing 0.8 ml of erythrocytes suspension to produce concentration of 2–165 μM. The samples were incubated for 30 min with gentle shaking at 37°. Preliminary distribution studies showed that an equilibrium between erythrocytes and the medium was achieved within 10 min and was constant for at least 1 hr.

Calculations. Several theoretical calculations were made to evaluate the amounts of indapamide bound to plasma proteins and to erythrocytes, respectively, as well as the amount of unbound drug in whole blood. According to the law of mass action, and assuming that $1/K \gg F$, we may write

$$B_i = (NK)_i \cdot F \quad (1)$$

$$B_e = (NK)_e \cdot F \quad (2)$$

in which B_i, B_e, and F are, respectively, the bound ligand concentration to each plasma protein of the ith class, the ligand concentration in erythrocytes, and the free ligand concentration. From mass balance considerations, the total ligand concentration (T) is

$$T = (1 - H) \cdot F + H \cdot B_e + (1 - H) \cdot \Sigma B_i \quad (3)$$

in which H is the hematocrit.

After substituting eqns (1) and (2) in eqn (3), eqn (3) becomes

$$T/F = (1 - H) \cdot (1 + \Sigma(NK)_i) + H \cdot (NK)_e \quad (4)$$

Then

$$f_e = H \cdot (NK)_e \cdot F/T \quad (5)$$

$$fB_i = (1 - H) \cdot (NK)_i \cdot F/T \quad (6)$$

where f_e and fB_i are, respectively, the fractional amount of ligand in erythrocytes and bound to each plasma binding protein.

RESULTS

Binding to serum proteins

The binding of indapamide to normal sera was 76.3 ± 1.0% (five sera). Table 1 shows the binding parameters of indapamide to isolated plasma proteins. It is mainly bound to AAG with a high association constant, and to a lesser extent to HSA and lipoproteins. The binding of indapamide to AAG is depicted in Fig. 2 and was assumed to occur via a single site, since a Fisher statistical test with the least-squares criterion showed that there was no benefit achieved by increasing the number of binding parameters above two (n and K). As the binding to HDL and LDL was unsaturable, no association constant or binding site concentration could be derived from these data. So, a total binding constant, nK, relating the bound to the free drug concentration was estimated.

Table 1. Parameters of indapamide binding to plasma proteins (±SD)

Protein	n	K (mM^{-1})	nK (mM^{-1})
HSA	0.94 ± 0.17	2.5 ± 0.6	
AAG	0.70 ± 0.03	73.4 ± 12.6	
HDL			9.2 ± 0.4
LDL	19.20 ± 5.7	3.8 ± 1.6	
VLDL			212 ± 7

Abbreviations: HSA, human serum albumin; AAG, α_1-acid glycoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

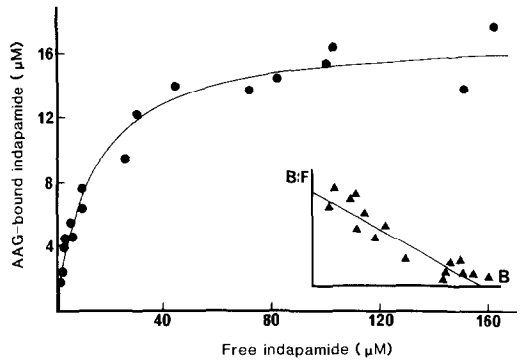

Fig. 2. Indapamide binding to 22.7 μM AAG. Insert: Scatchard plot.

Binding to erythrocytes and platelets

Indapamide was extensively bound to erythrocytes (f_e between 90 and 96%) suspended in PBS (H = 0.48). The binding was ascribed to a saturable (N = 57 ± 22 μM, K = 385 ± 283/mM), plus a non-saturable process (NK = 9.4 ± 0.9). The binding to erythrocytes was slightly diminished in whole blood (H = 0.48, f_e between 81 and 93%), and the binding parameters relating erythrocytes and plasma indapamide concentrations were diminished (K = 13 ± 3/mM, N = 639 ± 91 μM). In both cases, the binding was saturable, but the relationship between erythrocytes and plasma concentrations could be considered as linear in the therapeutic range (Fig. 3).

In order to examine the effect of the binding proteins on the erythrocytes-to-serum partitioning, erythrocytes were resuspended (H = 0.35) in PBS containing variable concentrations of AAG or HSA.

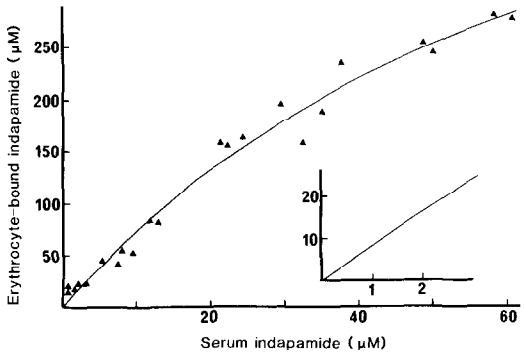

Fig. 3. Erythrocytes-to-plasma partitioning of indapamide. Insert: therapeutic range of whole blood concentrations.

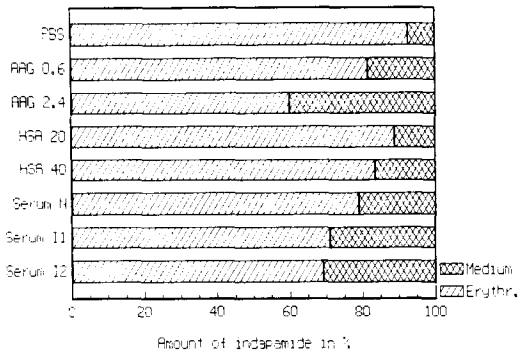

Fig. 4. Distribution of indapamide between erythrocytes and the suspending medium (protein solutions or sera). Protein concentrations in g/l. N, I1, I2: normal and two inflammatory sera (HSA, N: 48, I1: 29, I2: 27 g/l; AAG, N: 0.37, I1: 2.60, I2: 2.58 g/l).

As shown in Fig. 4, AAG is more effective than HSA to lower the f_e. In parallel experiments, erythrocytes were resuspended in either normal or sera from patients with inflammatory syndrome. Although inflammatory sera had a net reduction in HSA concentration (29 and 27 g/l vs 48 g/l), they were more effective than the normal serum to decrease the amount of indapamide associated to erythrocytes (f_e: 71 and 69% vs 80%) because of their higher concentrations of AAG (2.60 and 2.58 g/l vs 0.37 g/l).

The binding of indapamide to platelets was negligible.

Simulations of blood distribution of indapamide

Using the estimated binding parameters of indapamide to the different blood fractions, we simulated the distribution of indapamide in blood at therapeutic level (200 ng/ml or 0.55 μM). Since indapamide was at least 80% bound to erythrocytes and 70% bound to serum proteins, the free indapamide concentration in blood should not exceed 10% of the total, that is 0.055 μM. This value was clearly lower than the $1/K$ values (dissociation constants) obtained with the binding components in blood (400, 13.5, 263 and 2.6 μM for HSA, AAG, LDL and erythrocytes, respectively), so the condition $1/K \gg F$ for eqns (1)–(6) was verified. Two possibilities were considered: the normal serum and the sera with biological syndrome of inflammation that were used above in the erythrocytes-serum partition experiments. As shown in Table 2, the calculated values are close to the measured values, but slightly higher. This is because the binding due to the lipoproteins was not taken into account in the calculation, since we did not determine the lipoprotein concentrations in these sera.

DISCUSSION

Acidic drugs are generally bound to serum to a great extent (>90%), and this is determined by a high-affinity interaction with HSA, the fraction bound to erythrocytes amounting to the free serum fraction of drug [5, 6]. By contrast, our results indicate that the major determinants of indapamide binding in blood are erythrocytes, and to a lesser degree, AAG.

This particular serum binding profile resembles that of basic drugs [5], and may be explained by at least two considerations: first, at physiological pH, indapamide is neutral, whereas nearly all acidic drugs are anions, and second, indapamide does not display a carboxylic moiety, thus can interact with AAG [7, 8]. The binding to AAG occurred via a single high affinity site, as currently observed with AAG-bound drugs [9, 10]. Also like basic compounds, indapamide was bound to HSA with a low association constant and to the lipoprotein fractions.

The high extent of indapamide binding to erythrocytes has been ascribed to an interaction with the carbonic anhydrase [2]. Other drugs such as chlorthalidone or acetazolamide are extensively associated to erythrocytes via a strong interaction with carbonic anhydrase [11, 12]. Saturable drug binding to erythrocytes can result in non-linear pharmacokinetic properties, since the erythrocytes-bound drug is thought not be readily exchangeable as shown for chlorthalidone [13]. In the therapeutic range of indapamide concentrations in whole blood, the relationship between whole blood and plasma concentrations is linear, hence non-linearity in inda-

Table 2. Comparison of computed and measured distribution of indapamide in blood (hematocrit = 0.35, indapamide blood concentration, 200 ng/ml or 0.55 μM)

Blood fraction	Erythrocytes resuspended in serum		
	Normal	Inflammatory 1	Inflammatory 2
Concentration of			
AAG (g/l)	0.37	2.60	2.58
HSA (g/l)	48.0	29.6	27.8
Computed % bound to			
AAG	2.4	15.0	15.1
HSA	8.6	4.7	4.5
erythrocytes	84.0	75.8	75.9
Measured % bound to erythrocytes	79.2	70.7	70.0

Abbreviations: HSA, human serum albumin; AAG, α_1-acid glycoprotein.

pamide pharmacokinetics is not expected to result from indapamide binding to erythrocytes. Although indapamide is extensively bound to erythrocytes, variations in serum binding (mainly due to variations in AAG concentration) can involve variations in the distribution of indapamide between erythrocytes and serum. Thus altered hematocrit and/or AAG concentrations can involve changes in erythrocytes/plasma partitioning of indapamide, resulting in altered pharmacokinetics.

Acknowledgements—The part of this study relevant to lipoproteins was supported by a grant from the 'Institut National de la Santé et de la Recherche Médicale (INSERM)', contrat de recherche externe No 867012.

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