In vitro amiodarone protein binding and its interaction with warfarin

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Summary. The binding of amiodarone to human plasma protein and to bovine serum albumin was studied by three different methods, ultracentrifugation, equilibrium dialysis and fluorescence spectroscopy. The fraction of amiodarone bound to plasma protein amounted to 96.3%. The changes in the binding properties of 1-anilino-naphthalene-8-sulfonate for bovine serum albumin using warfarin and amiodarone as independent inhibitors were analyzed in terms of binding site specificity. The findings indicated that amiodarone and warfarin have two different binding sites on bovine serum albumin, so a noncompetitive inhibition mechanism was indicated. On the basis of our data we cannot exclude other mechanisms of interaction besides direct displacement of one drug by another; nevertheless, metabolite interference between amiodarone and coagulation cofactors may better explain the enhancement of warfarin's pharmacological action in association with amiodarone.

Key words. Amiodarone; warfarin; protein-binding; drug interaction; fluorescent probes; human plasma protein; bovine serum albumin.

Amiodarone, an iodinated benzofuran, controls a wide range of tachyarrhythmias refractory to conventional treatment[4-6]. However, no agreement has been reached on the optimal dose schedule, nor has any precise estimate been made of the incidence of adverse reactions associated with an effective maintenance dose. Amiodarone's unique pharmacokinetic properties have attracted the attention of both clinicians and pharmacologists[7-9] who have attempted to link its long half-life (days after chronic treatment), its large volume of distribution and its accumulation in selected tissues to its clinical effects. Although the relationship between the drug and its side effects is not yet clear, selective accumulation of the drug might explain the lung fibrosis and corneal spots[10], but not all the variable effects on thyroid metabolism[11].

With these questions in mind we studied the noncovalent binding of amiodarone to plasma protein, one of the still disputed keys in the complex pharmacology of the drug[7]. Two classical methods were applied; ultracentrifugation sedimentation and equilibrium dialysis. In addition, to investigate the drug interaction recently reported between amiodarone and warfarin[12,13], we studied the effects of the two drugs on the binding of 1-anilino-napththalene-8-sulfonate (ANS) to bovine serum albumin (BSA) by fluorescent spectroscopy.

Materials and methods. Amiodarone hydrochloride was synthesized, purified and supplied by Labaz (Centre de Recherche, Brussels, Belgium). All other chemicals were reagent grade from commercial sources. Phosphate buffer 0.067 M, pH 7.4, 0.9% NaCl was used in all experiments. BSA fraction V was obtained from Sigma, St Louis, Mo, USA.

Amiodarone concentrations were determined by high performance liquid chromatography[14].

Ultracentrifugation. Amiodarone was added to plasma from healthy volunteers at 37 °C under magnetic stirring to give a concentration of 10 µg/ml, in accordance with the method of Aarons et al.[15]. Four 0.4-ml fractions (P1, P2, P3, P4) were collected and the concentrations measured. In each experiment a sample containing the drug dissolved in buffer was prepared as control.

Equilibrium dialysis. Experiments were carried out using a Dianorm apparatus according to Weder et al.[16], and the system was equilibrated overnight at 37 °C in a thermostatic bath. The drug was assayed in the two chambers of the dialysis cells. Dialysis membranes rinsed with methanol were checked for nonspecific binding.

Fluorescence spectroscopy. 1-Anilino-naphthalene-8-sulfonate (magnesium salt) (ANS) was prepared according to Weber and Young[17], and the extinction coefficient of 5.01×10^3 M^{-1} cm^{-1} was calculated at 350 nm to test the purity of the compound. Fluorescence was measured on a Shimatzu RF-510 spectrofluorimeter at 28 °C exciting at 388 nm and the fluorescence emitted was observed at 475 nm, using 1 cm path length quartz cuvets and 5 nm excitation and emission slits. ANS was titrated manually using a Hamilton syringe, adding saturating concentrations of the two drugs (1.8×10^{-4} M), separately, to the cuvet before the beginning of the experiments in presence of amiodarone and warfarin.

Calculations and results. Evidence that amiodarone might bind to plasma protein to a high degree has been put forward by Andreasen et al.[18]. Here we present our findings from ultracentrifugation, equilibrium dialysis and fluorescence spectroscopy.

Ultracentrifugation. The fraction of amiodarone bound to plasma protein calculated as the ratio between the deepest fraction collected from the centrifuge tube (P4) and the total concentration of the drug in solution (P1 + P2 + P3 + P4) gave a value of 96.3%. In the sample where amiodarone was dissolved in protein-free buffer, partitioning was observed in the superifical fractions P1 and P2.

Equilibrium dialysis. Though equilibrium dialysis can be considered as a closest model close to physiological conditions, some difficulties arose with this technique in calculating the amount of amiodarone bound to plasma proteins:

a) the low solubility of the drug in buffer solution[19];

b) the long time needed to achieve equilibrium between the two sides of dialytic cells (more than 24 h);

c) the high nonspecific binding of the drug to the dialysis membrane (30–35%).

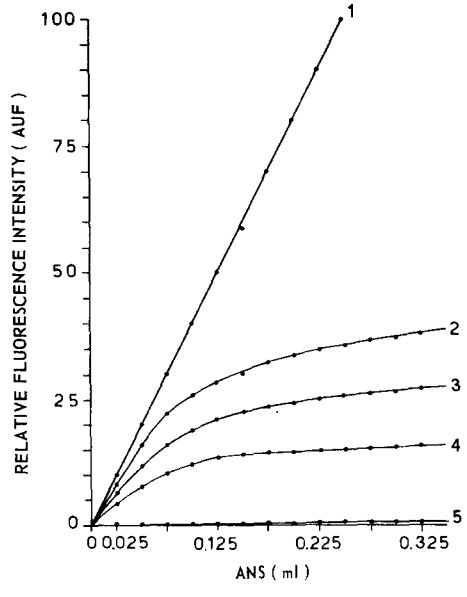

Figure 1. Fluorescence titration with ANS. The equilibrium solution contained 3.5×10^{-5} M (1), 3.5×10^{-6} M (2), 2.5×10^{-6} M (3), 1×10^{-6} M (4) and no (5) BSA, in a total volume of 2 ml at pH 7.4. From 0 to 0.325 ml of a 2.13×10^{-4} ANS solution were added.

This technique was therefore not acceptable for calculating amiodarone-protein binding.

Fluorescence spectroscopy. The enhancement of fluorescence observed when some aromatic dyes bind to protein can be used to measure the stoichiometry and equilibrium constants of the dye-protein complex. Since ANS appears to behave in this way[17,20,21], we used this method to study the systems amiodarone-BSA and warfarin-BSA.

The fraction of dye bound, X, was computed as:

$$X = ((F_{obs}/F_{free}) - 1)/((F_{bound}/F_{free}) - 1). \quad (1)$$

In this expression F_{free} and F_{bound} refer to the fluorescence intensities recorded when all the dye in the solution is respectively free and bound. The lower curve (No. 5) in figure 1 represents the titration of ANS in phosphate buffer and gives the values of the free dye fluorescence as a function of ANS concentration. The upper curve (No. 1) shows the same titration in the presence of 3.5×10^{-5} M BSA, at which concentration essentially all the ANS molecules are bound. Curves 2, 3 and 4 were obtained at intermediate protein concentrations to give the F_{obs} values, which contain the information relative to the association constant.

The average of number of dye molecules bound per mole of protein (v) was calculated as:

$$\bar{v} = X\ (At)/(Pt), \quad (2)$$

where (At) = total dye concentration and (Pt) = total protein concentration.

Experimental points were plotted according to Scatchard[22]. If amiodarone and warfarin also bind to protein at the same binding sites as ANS, their relative association constants K_b can be calculated in accordance with Klotz et al.[23]:

$$K_b = K_a(A)/(PA)/n(Pt)K_a(A) - K_a(A)\ (PA) - (PA)/(Bt)K_a(A) - n(Pt)K_a(A) + K_a(A)(PA) + (PA) \quad (3)$$

This equation describes simple competition by two ligands for equal and independent binding sites on a macromolecule where K_b = the association constant for the competitor (drug), K_a = the association constant for the ligand A (ANS), (A) = concentration of free A, (PA) = concentration of bound A, n = number of binding sites, (Pt) = total protein concentration, and (Bt) = total concentration of the competitor. In this case n, the number of the binding sites ($\bar{v}_A$ on the horizontal axis of the Scatchard plot), will be the same as in the absence of the competitor. Alternatively, if the drug does not compete for the same site, experimental points will reveal different numbers of binding sites.

The experimental points in the presence of warfarin (fig. 2a) and amiodarone (fig. 2b) were fitted, and in the same system of axes the results for the simple binding of ANS to BSA are shown. We calculated a K_a for ANS of 2.8×10^5 M^{-1} and the number of sites was 5.11 ± 1.25, in good agreement with Weber and Young[17].

Warfarin was found to compete for the same binding sites as ANS and a K_a of 1.9×10^4 M^{-1} was calculated from equation 3. In the presence of amiodarone different binding sites were found for ANS. In this case we cannot use Klotz's equation so we can give no K_a value for amiodarone.

Discussion. The aims of the experiments described were, first, to determine the extent of amiodarone binding to plasma protein and, second, to provide evidence that the amiodarone-warfarin interaction can be explained in terms of binding site displacement.

To answer the first question we looked for a method that offered good reproducibility and had been widely used by other investigators; equilibrium dialysis was selected. Unfortunately the high nonspecific binding to the dialysis membrane, and the long time needed to reach osmotic equilibrium, greatly reduced the utility of this technique. To calculate the fraction of amiodarone bound, we applied the sedimentation method by ultracentrifugation and the high value obtained, in good agreement with Andreasen et al.[18], is associated with the hydrophobic nature of amiodarone, its low solubility in aqueous solution and the significant role of protein as a solubilizing agent. However, knowledge of the percentage bound by itself merely indicates the drug's ability to bind to proteins and gives no information about binding constants. To investigate whether the ability of both amiodarone and warfarin to bind to protein at the same site might be the cause of their pharmacological interaction we studied the effects of the two drugs separately on the ANS-BSA complex. Probably, if amiodarone and warfarin had had the same binding sites, we could have calculated the still unknown K_a for amiodarone and compared it with that for warfarin[24]. Figure 2b clearly shows that this was not the case: while warfarin does bind to BSA at the same site as ANS, amiodarone does not.

In the spectroscopic study we considered BSA as a general model for the binding of a small molecule to proteins. It is possible that the two drugs behave slightly differently in vivo. However, because our findings for warfarin with BSA closely resembled those obtained by Jun et al.[24] with human serum albumin, we feel that BSA probably represents a satisfactory approximation, as a general model for studying binding site interaction.

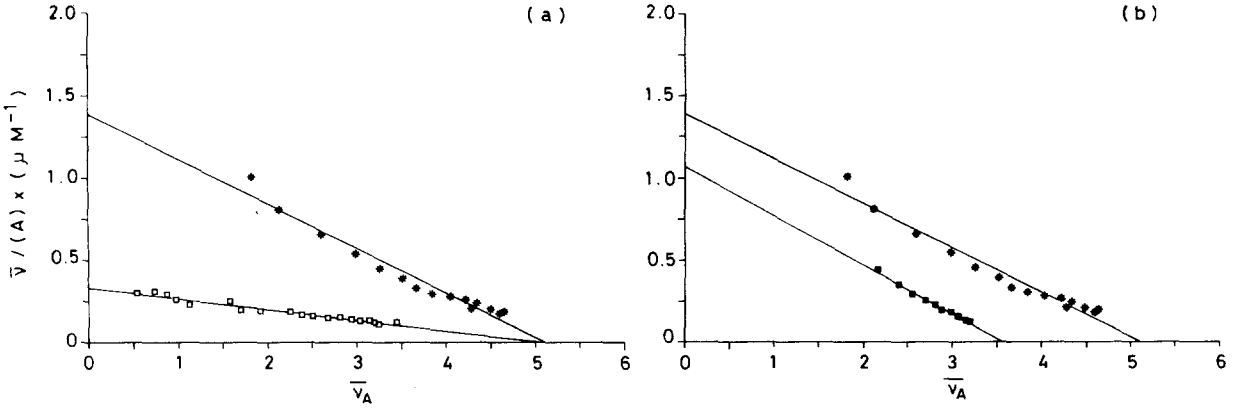

Figure 2. Scatchard plots of ANS-BSA complex (*) in the presence and absence of sodium warfarin (a, □) and amiodarone (b, ■). The experimental curves for the ANS-BSA complex in the absence of the drug drawn in *a* and *b* are the same, for simplicity. For every real competition experiment the same titration was repeated in the absence of the drug.

In conclusion, although this report confirms the large fraction of amiodarone bound to proteins found by other investigators[18], it also strongly supports the suggestion that the reduced doses of oral anticoagulant required when patients also receive amiodarone is not due to direct displacement from the protein binding sites[25]; it might result from increased catabolism of vitamin K-dependent clotting factors[13,26], as an effect of the metabolic changes and thyroid disorders induced by amiodarone.

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Effect of mycotoxin (T-2 toxin) on catecholamine and Na^+, K^+-ATPase levels in rat epididymis

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Summary. The effect of mycotoxin (T-2 toxin) on catecholamines and Na^+, K^+-ATPase activities in rat epididymis has been evaluated. Dopamine and norepinephrine levels were significantly elevated in the caput and corpus regions whereas their levels remained unchanged in the caudal part of the epididymis. Na^+, K^+-ATPase activity was significantly increased in all the three regions of rat epididymis as a result of the toxin treatment. These changes may suggest an adverse effect on epididymal functions in rats.
Key words. T-2 toxin; catecholamine; ATPase; rat epididymis.

The epididymis plays an important role in mammalian reproduction because spermatozoa acquire their ability to fertilize the ovum during their passage from the caput to the caudal region of the epididymis[1]. Thus, the epididymis provides a suitable biochemical environment for the maturation of spermatozoa[2]. The physiological functions of the epididymis are precisely regulated by androgenic hormones, intracellular enzymes and other biochemical constituents[3]. Amongst the trichothecene group of mycotoxins, T-2 toxin has been reported to cause a variety of changes such as reduced viability of spermatozoa, aspermia and degenerative changes in the testes of animals, which affect the male reproductive function adversely[4]. These changes have been reported in the testis, but the effect of T-2 toxin on the biochemistry of epididymis has not been described so far. In the present study, therefore, we investigated the effect of T-2 toxin on the levels of catecholamines, Na^+, K^+-ATPase activity in all the three anatomical regions of the rat epididymis.

Materials and methods. Norepinephrine (NE), dopamine (DA), opthalaldehyde, adenosine triphosphate (ATP), ouabain and 1,2,4-aminonaptholsulfonic acid (ANSA) were obtained from Sigma Chemicals Co., USA. All chemicals used were of analytical grade.

Male albino rats weighing about 150 g each from the Laboratory Animal Research Division of this institute were given T-2 toxin (1.25 mg/kg b.wt) by intubation for five consecutive days orally. Symptoms such as hair erection and loss of b.wt. were observed as a result of the toxin treatment. On the sixth day the animals were sacrificed by decapitation. Epididymes were collected and divided into different segments with scissors in the cold.

Na^+, K^+-ATPase assay. ATPase was assayed by the procedure

Table 1. Effect of T-2 toxin on Na^+, K^+-ATPase of rat epididymis

Treatment	n	Caput	Corpus	Cauda
Control	6	44.66 ± 3.41	51.80 ± 3.26	49.68 ± 3.70
Toxin-treated	6	61.84 ± 3.97***	73.00 ± 4.31***	61.50 ± 4.49

Values are mean ± SE expressed in μg/h/mg protein. ***$p < 0.01$.