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SHORT COMMUNICATIONS

Effects of pressure on the plasma binding of digoxin and ouabain in an ultrafiltration apparatus

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THE PLASMA binding of drugs determines, in part, their onset of action, metabolism and rate of excretion. Drugs that are extensively bound tend to have prolonged half-lives *in vivo*.[1] Digitoxin, digoxin and ouabain have long (14–21 days), medium (2–6 days) and short (1–3 days) durations of action respectively.[2,3] Doherty and Hall[3] used the method of gel filtration to study digoxin binding. They concluded that digoxin, a more polar molecule than digitoxin, was not bound to serum proteins and suggested that this lack of binding of digoxin explained, in part, the difference in the serum half-life of digoxin and digitoxin. However, ouabain, which is also thought to be unbound,[4,5] has a significantly more rapid speed of onset and a shorter plasma half-life than digoxin.[2] Nevertheless, Doherty and Hall[3] do state that "the technique used in this (their) study did not necessarily exclude the possibility of a 'weakly bound' compound being separated by manipulation attendant to the experiment." These same investigators[3] also employed equilibrium dialysis to measure digoxin binding in the serum, but were unable to attain equilibrium at 96 hr. Moreover, they raised the possibility of membrane adsorption of digoxin. For these reasons, they questioned the validity of this method for measuring digoxin binding. However, Lukas and De Martino[6] found digoxin was 23 per cent bound to human plasma at a total concentration of 5 ng/ml by the equilibrium dialysis technique.

In view of the disparate results for digoxin binding, the problem of digoxin and ouabain binding to plasma proteins was reinvestigated using different methodology and plasma digoxin concentrations in the high therapeutic–low toxic range.[7–9] In this study we have employed two modifications of a rapid method used to study drug binding to plasma proteins.[10] This report confirms the binding of digoxin to plasma proteins,[6] and demonstrates an effect of pressure on drug binding in an ultrafiltration apparatus.

Tritiated digoxin and tritiated ouabain of specific activity 12 and 13 Ci/m-mole respectively (New England Nuclear Corp., Boston, Mass.), were diluted to a final concentration of 6·5 ng/μl with ethanol–water (50:50), termed the "glycoside stock solutions." To ascertain the purity of the radiodigoxin, the radiolabeled digoxin and unlabeled digoxin (Sigma Chemical Company, St. Louis, Mo.) were chromatographed on Silica gel G thin-layer chromatography plates in an ascending system using ethyl acetate–methanol–water (80:5:5) as solvent. The plates were scraped at 0·5-cm intervals and the radiolabeled gel samples counted in Aquasol (New England Nuclear Corp.) by standard scintillation methods.[11] The Silica gel samples containing the unlabeled digoxin were eluted with ethanol–H_2O (50:50), and the absorbance of the eluate was read at 231 nm in a Gilford spectrophotometer. The radioactivity was present in a single peak representing 94 per cent of the total radioactivity, and corresponded to the R_f of the unlabeled digoxin standard.

To attain economy of sample, a 2·5-ml plastic chamber with a dead space of 0·1 ml below the 13-mm Millipore filter (PSED0130; Millipore Corp., Bedford, Mass.) was employed. By using Henry's law, a pH of approximately 7·4 was maintained in the parent plasma solution by adjusting the concentration of CO_2 in the applied gas. A 2·5-ml sample of parent plasma or salt solution containing glycoside was added to the cleaned, dry chamber. The chamber was then capped and a pressure hose coming from a compressed gas tank (*vide infra*) was attached to the cap. A pressure of from 0·1 to 45 pounds per square inch (p.s.i.) was applied to the chamber and the magnetic stirrer was set at approximately 200 rev/min. The filtrate passed into the base of the apparatus to the outside where 0·1-ml aliquots of filtrate were collected. The filtration of 0·6 ml of parent plasma solution required approx. 40 min for completion at 45 p.s.i. After each filtration, the filter was discarded and the apparatus was washed, rinsed in distilled water, and dried thoroughly.

Recovery of digoxin or ouabain in the filtrate in the absence of protein was determined by the addition of the glycoside stock solution to balanced salt solution[12] to yield final concentrations of 0·7, 1·3 and 2·6 ng/ml (henceforth called the parent salt solution). The parent salt solution was bubbled with 5% CO_2 for 5 min and forced through the filter at zero, 0·1 and 2·0 p.s.i. using 5% CO_2 (95% N_2). Six 0·1-ml aliquots of the filtrate were collected at 23°. The filtration of 0·6 ml required

approx. 2 hr at zero p.s.i., 40 min at 0·1 p.s.i. and 10–15 min at 2·0 p.s.i. Duplicate aliquots were counted from each fraction as well as from the parent solution before and after the filtration.

To determine plasma binding, fresh heparinized plasma was obtained from two healthy male donors. Immediately after centrifugation, 2 ml plasma was added to 0·5 ml of balanced salt solution[12] and 1 μl of either the ouabain or digoxin stock solution, which yielded a final concentration of 2·6 ng/ml (the parent plasma solution). The mixture was gently shaken and allowed to sit at room temperature for 30 min or longer, but never exceeding 90 min. After incubation, the parent plasma solution was filtered at 23° with 1·41% CO_2, the balance nitrogen, at 45 p.s.i. The use of 45 p.s.i. necessitated the reduction in CO_2 content to 1·41 per cent to maintain the pH in the parent plasma solution at approximately 7·4. The first 0·5 ml of filtrate was discarded, and the next 0·15 ml of filtrate was collected. Two 50-μl aliquots were counted from the 0·15-ml filtrate aliquot and the parent plasma solution at the end of the filtration. The fraction of free digoxin and ouabain was calculated after correction for quench and nonspecific binding by the filter (*vide infra*) by dividing the dis./min in the filtrate by the dis./min in the parent solution taken at the end of the filtration. Similar experiments were performed at 30, 15 and 2 p.s.i. with appropriate increase in the CO_2 content to keep the pH approx 7·4.

Experiments were also performed to measure digoxin binding exactly as above, except the following were individually varied: concentration was increased to 1 μg/ml, and serum was employed rather than plasma.

Ouabain quantitatively passed through the filter at 1·3 and 2·6 ng/ml in balanced salt solution. Digoxin, however, did not. This was due to nonspecific binding by the cellulose base on which the filtering membrane rested.[10] Thus, at a total digoxin concentration of 2·6 ng/ml in the parent salt solution, after 0·5 ml of the parent solution was filtered and discarded, the concentration of digoxin in the subsequent 0·1-ml filtrate aliquot was 93·0 $\pm$ 1·4% (S.E.M.) that of the parent salt solution. Hence, the amount of digoxin passing through the filter needed to be corrected for the small amount of nonspecific binding.

In Fig. 1 is plotted the percentage of unbound digoxin at a total digoxin concentration of 2·6 ng/ml (plasma) versus pressure at 23°. The straight line was obtained by the method of least squares and the parallel dashed lines are the 95 per cent confidence interval for the extrapolated value of unbound digoxin (76·1 per cent) at zero applied pressure.[13] Altering concentration (from 2·6 ng/ml to 1 μg/ml), binding medium (serum) or incubation time did not change significantly the amount of digoxin bound.

The extrapolated value of unbound ouabain at zero pressure at 23° is 97·5 per cent (see Fig. 1).

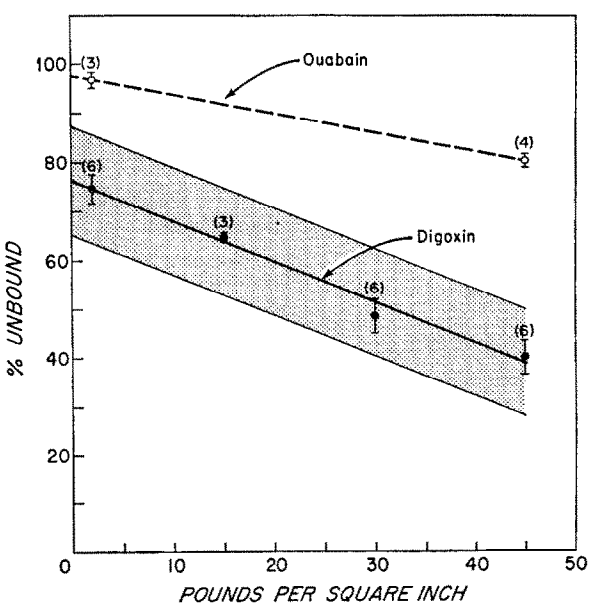

FIG. 1. Plasma binding of digoxin and ouabain vs pressure at 23°. The concentration of both ouabain and digoxin is 2·6 ng/ml. Errors are standard errors of the mean. The numbers in parentheses indicate the number of experiments at each point.

Our finding of an increased apparent binding of digoxin and ouabain at higher pressures was unexpected. This effect does not occur with salicylate at 0·1 and 1·0 mM concentration.[11,*] The exact explanation for this effect is uncertain, but might be due to partial blocking of the filter pores by plasma proteins or an increased concentration of plasma protein immediately above the filter at higher pressures and, hence, higher filtration rates. Pressures of 45 p.s.i. (3 atm) should not affect the digoxin binding in the bulk of the parent solution.[14] We speculate that this effect of pressure on the binding of substances may lead to artifacts (e.g. in ultracentrifugation studies), but we have no data on this point.

Extrapolation of the binding to zero pressure should eliminate the artifacts of pressure. The extrapolated value of 76·1% digoxin unbound is in close agreement with the 77 per cent value reported by Lukas and De Martino.[6] Increasing the concentration to 1 μg/ml of digoxin did not change the binding. This was also noted by Lukas and De Martino.[6] However, our results are at variance with those of Doherty and Hall.[3] We believe this disparity in results is explained by Cooper and Wood,[15] who state that gel filtration "is reliable only if the protein–ligand complex dissociates at a rate which is low compared with the rate of elution. Failure to appreciate this result could lead to misleading results." Doherty and Hall[3] are aware of the "possibility of a 'weakly bound' compound being separated" from its binding site in the process of gel filtration. Our method, using Henry's law to control the pH, and pressure and slow rotational stirring to ultralfiter the sample, is able to detect this weak binding.

These experiments support the hypothesis[3,15,16] that the more polar glycoside, ouabain, is less bound to plasma proteins than digitoxin, a relatively nonpolar glycoside which is extensively bound.[6] Digoxin lies between ouabain and digitoxin in both plasma binding and polarity. Thus, plasma binding might explain, in part, the differences in the speed of onset of action of these glycosides as well as the half-life of these cardiac glycosides in the body.[1,2]

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* R. Spector, unpublished observations.