SOME CLINICAL IMPLICATIONS OF THE PROTEIN BINDING OF DIGOXIN

SUSAN WALLACE & B. WHITING

Department of Materia Medica, University of Glasgow, Stobhill General Hospital, Glasgow G21 3UW

- 1 The binding of [^3H]-digoxin to human plasma proteins was studied using both ultrafiltration and frontal analysis, to establish a suitable method for the measurement of digoxin-protein interaction, and to assess the effect of variations in digoxin protein binding on its distribution.
- 2 Using ultrafiltration, [^3H]-digoxin was found to bind to plasma proteins to the extent of 30-40% over a concentration range of 0.001-1 ng/ml. Frontal analysis was unsuccessful in the measurement of digoxin protein binding.
- 3 Dilution experiments using plasma and human serum albumin suggested that variations in digoxin protein binding would be unlikely to cause significant alterations in the levels of free drug in the body.
- 4 The possible clinical implications of these results are discussed.

Introduction

The observation that cardiac glycosides bind to plasma proteins was first made by Oppenheimer in 1913. He showed that the effects of cardiac glycosides on the isolated frog heart were markedly reduced when the drugs were mixed with serum instead of Ringer solution. It is now clear that digitoxin is bound to human and animal serum albumin to the extent of 80-90%, and that this plays an important part in its kinetics (Lendle & Pusch, 1935; Fawaz & Farah, 1944; Farah, 1945; Rothlin & Kallenberger, 1950). The situation is not so clear, however, with digoxin, and little work has been carried out on the possible clinical significance of the plasma protein binding of this drug.

The various methods used to investigate digoxin protein binding have produced conflicting results. Three studies utilized Sephadex gel filtration techniques. Kuschinsky (1969) reported 20% binding of digoxin to serum and albumin solutions; Doherty & Hall (1970) found only 2% binding to plasma proteins, while Evered (1972) found 20-30% binding.

Equilibrium dialysis and ultrafiltration have also been used to investigate cardiac glycoside protein binding. Lukas & de Martino (1968) found 23% binding using equilibrium dialysis, but Doherty & Hall (1970) found that equilibrium could not be reached in their system even after 96 hours. The only study performed using ultrafiltration (Spector, Vernick & Lorenzo, 1973) reported 24% binding for digoxin.

Because there is some variation in these results, the present study was carried out to establish a satisfactory method for the measurement of digoxin-protein interaction, and to investigate the effects of variations in digoxin protein binding on its distribution.

Methods

Both an ultrafiltration method and a gel filtration technique were used to measure digoxin protein binding.

Ultrafiltration

Centriflo membrane cones with conical tubes (Amicon Corporation) were used, and were found to give rapid and reproducible results.

Human plasma was obtained from the blood bank, and human serum albumin in two batches from Koch-Light and Pentex. Tritiated digoxin ([^3H]-digoxin) (Radiochemical Centre, Amersham) was used, to facilitate measurement of the drug; the [^3H]-digoxin was added *in vitro* to the protein solutions to give final concentrations ranging from 0.001-1 ng/ml. Dilutions were made using Sørensen's phosphate buffer, M/15, pH 7.4. Albumin concentrations ranged from 1-5 g/100 ml in the pure albumin solutions. The final concentration of plasma after addition of [^3H]-digoxin in buffer was 80%. Aliquots (5 ml)

of the drug-protein solutions were incubated at room temperature for 10 min before being transferred to the Centriflo cones. The cones were presoaked in distilled water for at least 1 h before use, and were tested for contamination by filtering distilled water through them and counting the ultrafiltrate in a liquid scintillation counter. Since each cone was used for several estimations, frequent checks for protein leakage were carried out by the addition of 25% salicylsulphonic acid to the ultrafiltrate.

The [^3H]-digoxin protein solutions were centrifuged for 30 min at 750 g in an MSE Major centrifuge and approximately 2 ml of ultrafiltrate was obtained. Assay of [^3H]-digoxin in the ultrafiltrates was carried out by mixing samples (1 ml) with Instagel liquid scintillant (10 ml; Packard) and counting in a liquid scintillation counter.

All experiments were carried out at room temperature, 22°C. The pH of the digoxin-protein solution was measured before and after centrifugation, and remained constant at 7.4.

Gel filtration

A frontal analysis technique developed by Cooper & Wood (1968) was used. A fine glass column, 15 cm long and 0.5 cm in diameter, was filled with Sephadex G-25 Medium gel (Pharmacia) which had been allowed to equilibrate for 16 h in Sørensen's phosphate buffer, M/15, pH 7.0. The column was allowed to settle overnight, and was then flushed through with buffer, delivered by a slow injection apparatus to the top of the column at a rate of 1 ml/5 min, for 1 hour.

Solutions of 80% plasma, made up in buffer, and solutions of 4% and 2% bovine serum albumin (Miles Laboratories) were used. [^3H]-digoxin (1 ng/ml) was made up in buffer or protein solution. Samples (10 ml) of pure protein solution,

pure drug solution or drug-protein solution were applied to the top of the column at a rate of 1 ml/5 min by slow injection, and eluted with buffer (10 ml). Samples of 5 or 10 drops were collected using an LKB Ultrarac automatic fraction collector with drop counter. The protein concentration was monitored using an LKB Uvicord continuous flow ultraviolet absorptiometer, and recorded on an LKB 6520 chopper bar recorder. Samples of eluate (0.5 ml) were mixed with Instagel (10 ml), and counted for radioactivity.

As before, all experiments were carried out at room temperature.

Results

Ultrafiltration

(1) *Retention of [^3H]-digoxin by the membrane cone.* When a solution of [^3H]-digoxin (1 ng/ml) in buffer was spun for 30 min at 750 g, all the radioactivity was recovered in the ultrafiltrate. There is therefore no retention of [^3H]-digoxin by the membrane cone under these conditions.

(2) *Protein binding of [^3H]-digoxin.* The results of experiments carried out using an 80% plasma solution with varying digoxin concentrations are shown in Table 1. Under the conditions of the experiment, [^3H]-digoxin binds to serum proteins to the extent of 30-40%. There is a good correlation ($r = 0.994$, $P < 0.001$) between the amount of digoxin which is protein bound, and the digoxin concentration, with no evidence of serum protein saturation. *In vivo* studies carried out in the therapeutic range of 1-2 ng/ml yielded similar results with binding values of 30-40%. These studies were carried out on two normal, healthy subjects, and involved ingestion of

Table 1 Binding of [^3H]-digoxin to an 80% plasma solution, with varying concentrations of [^3H]-digoxin.

Digoxin (ng/ml)	% free digoxin	% bound digoxin	Concentration of free digoxin (ng/ml)	Concentration of bound digoxin (ng/ml)
1.000 (3)	66.5 $\pm$ 6.4	33.5 $\pm$ 6.4	0.67 $\pm$ 0.06	0.33 $\pm$ 0.09
0.330 (2)	54.4 $\pm$ 2.5	45.6 $\pm$ 2.5	0.18 $\pm$ 0.01	0.15 $\pm$ 0.01
0.100 (5)	60.6 $\pm$ 1.2	39.4 $\pm$ 1.2	0.06	0.04
0.033 (2)	62.7 $\pm$ 3.0	37.3 $\pm$ 3.0	0.02	0.009
0.010 (5)	68.9 $\pm$ 3.1	31.1 $\pm$ 3.1	0.007	0.003
0.003 (2)	64.2 $\pm$ 7.8	35.8 $\pm$ 7.8	0.002	0.001
0.001 (5)	80.3 $\pm$ 5.8	19.8 $\pm$ 5.9	0.0008	0.0002

Figures in brackets represent the number of experiments. Each value $\pm$ s.d. where applicable.

Fig. 1 Binding of [^3H]-digoxin to human serum albumin at varying concentrations of albumin. The concentration of [^3H]-digoxin is 1 ng/ml. (■) Percentage binding of [^3H]-digoxin in a solution containing the concentration of albumin found in a normal subject; (▲) % binding of [^3H]-digoxin in a 2% albumin solution, as might be found in a subject with hypoalbuminaemia. $r = 0.981$; $P < 0.001$.

[^3H]-digoxin in alcohol by mouth, and subsequent collection of venous blood samples over a period of 10 days. All samples used in binding studies were taken on the first day after ingestion of [^3H]-digoxin.

(3) *Effect of plasma dilution on [^3H]-digoxin protein binding.* A good correlation ($r = 0.968$, $P < 0.001$) was found between percentage plasma and percentage free [^3H]-digoxin. A difference of 10% was observed in the percentage free drug between the lowest (20%) and the highest (80%) plasma concentrations.

(4) *Effect of human serum albumin dilution on [^3H]-digoxin protein binding.* The range of concentrations of albumin used was typical of that found in man in health and disease. The results are presented in Fig. 1. Also shown are percentage binding figures which might be expected in a healthy subject with normal serum albumin (■), and in a subject with pronounced hypoalbuminaemia (▲). Maximum binding of [^3H]-digoxin to normal serum albumin was 20%, as opposed to 30–40% in whole plasma solution.

Gel filtration

Frontal analysis proved unsuccessful in the measurement of [^3H]-digoxin protein binding. Neither drug nor protein was retained by the gel, and the pattern for elution of digoxin remained the same, whether the drug was dissolved in buffer or plasma. The same system has, however, been shown by us to be successful in the measurement of protein binding of both [^{14}C]-warfarin and methyl red (Wallace & Whiting, unpublished observations).

Discussion

Ultrafiltration has been established as a rapid and quantitative method for the measurement of drug–protein interactions. The *in vitro* studies reported here indicate that under the experimental conditions outlined, [^3H]-digoxin is bound to human plasma to the extent of 30–40%, while binding to a 4% solution of human serum albumin is only 20%. Evered (1972) has pointed out that the presence of plasma lipid is evidently important in digoxin protein binding, and that binding in lipid-extracted serum and purified serum albumin solutions is reduced. It seems likely that the human serum albumin used in these experiments contained less lipid than normal plasma, and consequently digoxin protein binding is lower.

The value of 30–40% binding in plasma is somewhat higher than that reported by Spector *et al.* (1973) for their ultrafiltration system. A disadvantage of any centrifugal ultrafiltration system is that protein molecules tend to become adsorbed onto the membrane surface, and may block the membrane pores. The passage of free drug molecules through the membrane could be obstructed, resulting in a lower percentage of drug in the ultrafiltrate. It is possible that this could account for the relatively high binding values found in these experiments.

Two reasons present themselves to explain the failure of frontal analysis to measure [^3H]-digoxin protein binding. The binding may be so weak that the manipulations involved in the experiment cause the drug–protein complex to dissociate. Alternatively, a different grade of gel may be required, to facilitate greater retention of the free drug. Further studies are in progress, to clarify the situation.

Evered (1972) has suggested that protein binding of digoxin may be an important factor in the transport, distribution and excretion of the drug. As digoxin binding appears to be relatively weak in comparison to that of many other drugs,

it seems unlikely that binding will have a significant effect on these mechanisms.

Displacement of a drug from plasma proteins, by competition from other drugs for the same binding sites, by a decrease in the amount of protein available or by a reduction in its capacity to bind the drug, may in certain instances have a profound effect on the distribution of that drug. For example, the well-documented competition between warfarin and phenylbutazone (Aggeler, O'Reilly, Leong & Kowitz, 1967) can, theoretically, lead to a temporary increase in free warfarin levels in the tissues and a potentiation of anticoagulant activity. Warfarin is, however, a highly protein bound drug, whose distribution is likely to be affected by even small changes in the degree of protein binding. Digoxin is only 30-40% bound, and the studies carried out using diluted plasma and serum albumin suggest that fairly marked alterations in protein concentration, resulting in perhaps a 10% decrease in bound digoxin, will not affect the levels of free, pharmacologically active drug to any significant extent. These results are in agreement with the views of Hasselblatt (1971).

From a consideration of the relationship between plasma volume and total body water, it can be deduced that for a highly protein bound drug like warfarin, over 80% of the total drug in the body is located in the plasma (Hasselblatt, 1971); for digoxin, the figure is less than 20%. Drug molecules which are not bound to plasma

proteins are freely distributed throughout the body water, and it can be shown that if only a very small decrease in the amount of warfarin bound to protein occurred, the concentration of free, pharmacologically active drug would rise significantly, potentiating its anticoagulant effect. However, even if digoxin protein binding could be reduced by as much as 20%, this would result in less than 2% increase in the free concentration in the tissues. It is very unlikely, therefore, that variations in the protein binding of digoxin, even if quite large, would have any adverse effects clinically. It should be borne in mind, however, that the use of plasma digoxin levels in designing individual dose schedules should take into account both free and bound concentrations of the drug, as interindividual differences in binding could be of importance. While free drug levels in two patients might be similar, a small difference in protein binding could result in different total plasma levels. Attempts to increase total plasma levels by increasing digoxin dosage might conceivably cause a critical increase in the free drug level, leading to toxicity.

In conclusion, the experiments reported here indicate that digoxin is not extensively protein bound. The point is emphasized that changes in the protein binding of drugs having large volumes of distribution, such as digoxin, are not paralleled by marked alterations in free drug concentrations, or a potentiation of the drug's pharmacological activity.

References

- AGGELER, P.M., O'REILLY, R.A., LEONG, L. & KOWITZ, P.E. (1967). Potentiation of anticoagulant effect of warfarin by phenylbutazone. *N. Eng. J. Med.*, **276**, 496-501.
- COOPER, P.F. & WOOD, G.C. (1968). Protein binding of small molecules: new gel filtration method. *J. Pharm. Pharmacol.*, **20**, 150-156 S.
- DOHERTY, J.E. & HALL, W.H. (1970). Tritiated digoxin XV, serum protein binding in human subjects. *Am. J. Cardiol.*, **28**, 326-330.
- EVERED, D.C. (1972). The binding of digoxin by the serum proteins. *Eur. J. Pharmacol.*, **18**, 236-244.
- FARAH, A. (1945). On the combination of some cardioactive glycosides with serum proteins. *J. Pharmac. exp. Ther.*, **83**, 143-147.
- FAWAZ, G. & FARAH, A. (1944). A study of the digitoxin binding power of serum and other soluble tissue proteins of the rabbit. *J. Pharmac. exp. Ther.*, **80**, 193-198.
- HASSELBLATT, A. (1971). Interactions of drugs at plasma protein binding sites. In: *Toxicological Problems of Drug Combinations*, Proceedings of the European Society for the Study of Drug Toxicity, **13**, 89-97.
- KUSCHINSKY, K. (1969). On the characteristics of the binding of cardiac glycosides by plasma proteins. *Arch. Exp. Path. Pharmacol.*, **194**, 388-398.
- LENDLE, L.E. & PUSCH, P. (1935). Ueber die Binding der Digitaliskörper an die Eiweisstoffe des Blutes. *Arch. Exp. Path. Pharmacol.*, **177**, 550-563.
- LUKAS, D.S. & de MARTINO, A.G. (1969). Binding of digitoxin and some related cardenolides to human plasma proteins. *J. Clin. Invest.*, **48**, 1041-1053.
- ROTHLIN, E. & KALLENBERGER, A. (1950). Ueber das Glycosidbindungsvermögen verschiedener Eiweissfraktionen des Blutes. *Arch. Int. Pharmacodyn.*, **81**, 520-529.
- SPECTOR, R., VERNICK, R. & LORENZO, A.V. (1973). Effects of pressure on the plasma binding of digoxin and ouabain in an ultrafiltration apparatus. *Biochem. Pharmacol.*, **22**, 2485-2487.

(Received April 16, 1974)