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The plasma kinetics of digoxin-specific Fab fragments and digoxin in the rabbit

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Abstract—The plasma kinetics of total and free digoxin, and digoxin-specific antibody fragments (DSFab) in rabbits which had been given [^{3}H]digoxin one hour before DSFab has been studied over a 5 day period. Injection of DSFab caused a 4- to 5-fold rise in total digoxin and reduced elimination half-life ($t_{\frac{1}{2}\beta}$), apparent volume of distribution at steady-state (V_{dss}) and systemic clearance (CL) by 40, 90 and 75% respectively. Early in the experimental period, DSFab reduced free digoxin concentration (measured by ultrafiltration) from 4·1 ng mL^{-1} to a minimum of 1·3 ng mL^{-1} at 15 min. However, the concentration had rebound to 2·5 ng mL^{-1} by 60 min. Subsequently, free digoxin fell to 0·63 ng mL^{-1} and remained relatively constant over a 7 to 90 h period. The distribution half-life, $t_{\frac{1}{2}\beta}$, V_{dss} and CL for DSFab (concentrations measured by enzyme-linked immunosorbent assay) were 0·3 h, 3·2 h, 185 mL kg^{-1} and 57 mL kg^{-1} h^{-1}, respectively. A considerable molar excess (about 5) of DSFab in the plasma was necessary to maintain minimum free digoxin concentrations. When the DSFab:digoxin molar ratio was less than 4 during the initial treatment period, free (toxicologically active) concentrations increased. With the elevation in total digoxin, however, an opposite situation appeared to apply. By 24 h the relatively short DSFab $t_{\frac{1}{2}\beta}$ meant that the plasma DSFab concentration was $<0{\cdot}05$ μg mL^{-1} giving a DSFab:digoxin molar ratio of below 0·06, yet the antibody-induced rise in total digoxin concentration was still detectable at 100 h.

A recent large-scale multicentre clinical study has confirmed the value of digoxin-specific antibody (Fab) fragments (DSFab), derived from sheep immunoglobulin G (IgG), in treating severe cardiac glycoside toxicity (Antman et al 1990). However, in spite of this established clinical usage some aspects of the effects of DSFab on hapten disposition are unclear. For instance, in two recent reports (Hursting et al 1987; Sinclair et al 1989), each involving the measurement of plasma digoxin concentrations in a patient being treated for digoxin poisoning, it was noted that DSFab administration rapidly reduced free (pharmacologically active) drug concentration by more than 95%. However, in blood samples taken 12 h later, it was found that the concentration of free digoxin had returned to 40–80% of the pretreatment levels, despite the fact that measurement of antibody concentrations revealed maintenance of a fourfold molar excess of DSFab in the plasma (Sinclair et al 1989). It is conceivable that this rebound phenomenon could be associated with a recurrence of clinical toxicity. The present study examines the rebound effect in the rabbit. Free and bound digoxin, and DSFab concentrations were measured in the plasma allowing the in-vivo stoichiometric relationship between DSFab and digoxin, as well as their plasma kinetics, to be investigated.

Materials and methods

Digoxin-specific Fab fragments (Digibind), lyophilized powder derived from anti-digoxin IgG raised in sheep, were received as gifts from the Wellcome Foundation Ltd, Beckenham, UK.

Unlabelled digoxin was obtained from Wellcome Foundation Ltd, Beckenham, UK. Randomly labelled 12α-[^{3}H]digoxin, (sp. act. 10·0 Ci mmol^{-1}) was obtained from Du Pont, New England Nuclear, Southampton, UK. The purity of the labelled digoxin was checked by thin-layer chromatography using commercial silica gel sheets (Eastman Chromatogram Sheet, Kodak 100 μm gel thickness) with chloroform:methanol (9:1) as the solvent system. The plate was scanned for radioactivity using a Radiochromatogram thin-layer scanner (Panax system E 0111/p7900A) and a single sharp symmetrical peak obtained corresponding to unlabelled digoxin. The quenched tritium standards were obtained from Amersham International, Amersham, UK. The liquid scintillation fluid (NE260) was supplied by New England Nuclear, Edinburgh, UK. The micropartition system (MPS-1, Amicon, mol. wt cut-off 5000 daltons) for ultracentrifugation was supplied by Amicon Ltd, Glos., UK. Standard enzyme-linked immunosorbent assay (ELISA) reagents for sheep polyclonal DSFab were obtained as described previously (Timsina & Hewick 1990). All other reagents were obtained from British Drug Houses, Dorset, UK and were of analytical grade, unless otherwise stated.

Methods. Preparation of [^{3}H]digoxin for injection. An appropriate volume of [^{3}H]digoxin (1000 μCi mL^{-1}, 78 μg mL^{-1} ethanol) was evaporated to dryness under nitrogen. Digoxin (Lanoxin, 250 μg mL^{-1}) was added and the solution diluted with saline (0·9% NaCl) such that the total amount of digoxin in the injection solution was 15 μg mL^{-1} (12·5 μCi mL^{-1}). The [^{3}H]digoxin solution was stored at −20°C until required.

Procedures in conscious rabbits. The same animals were employed for digoxin alone (control) and digoxin/DSFab experiments. Female New Zealand White rabbits (3·2–5·1 kg) with free access to food and water were used. In control experiments, each animal was injected with [^{3}H]digoxin (12·5 μCi kg^{-1}, 15 μg kg^{-1}) via the left ear marginal vein and blood samples were taken from the right ear marginal vein at 5, 10, 15, 30 and 60 min and then every hour until 8 h and then at 24, 28, 32, 48, 51, 54, 56, 72, 76, 80, 96, 100 and 103·5 h. Plasma samples were obtained by centrifuging blood samples at 3000 *g* for 15 min and were stored at −20°C until the time of assay.

In digoxin/DSFab experiments, 60 min before DSFab dosing the rabbits were injected with the same dose of [^{3}H]digoxin as used in the control experiments, and an identical blood sampling time-table was employed. The rabbits then received a bolus i.v. injection of DSFab (1·9 mg kg^{-1}), this being the dose estimated to be twice-molar to the amount of digoxin present in the body just before the DSFab administration (the estimation of this dose is considered further under "Pharmacokinetic and statistical analysis"). Subsequent blood samples were taken at 5, 10, 15, 30, 45 min and 1 h and then every hour until 7 h and then at 24, 28, 32, 48, 51, 54, 56, 72, 76, 80, 96, 100 and 103·5 h. Plasma samples were obtained as described above.

There was a four week interval between control and digoxin/DSFab experiments. With two rabbits the control procedure was carried out first, while the other three rabbits were treated initially with digoxin/DSFab.

Determination of total and free [^{3}H]digoxin. For total digoxin, plasma samples (0·05 mL) were mixed with 5 mL liquid

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scintillant and the radioactivity counted using a Packard Tricarb 300 liquid scintillation counter. The counts min^{-1} were converted to d min^{-1} by reference to an efficiency vs channels-ratio curve for quenched standards. All determinations were carried out in triplicate.

For free digoxin, plasma samples (0·5 mL) were ultracentrifuged (1500 *g*, 20°C, 25 min) using the micropartition system (MPS-1). The ultrafiltrate (0·05 mL) was mixed with 5 mL liquid scintillant and the radioactivity corresponding to the concentration of free digoxin was measured in the same way as for total digoxin.

Free digoxin is used to refer to digoxin which is unbound to plasma proteins or DSFab. Plasma concentrations for both total and free [^{3}H]digoxin are expressed as ng mL^{-1} digoxin, as calculated from the specific activity of the injected solution, but are strictly speaking digoxin equivalents, as digoxin metabolites could be present.

Determination of DSFab concentrations. DSFab concentrations were determined by an ELISA based on anti-sheep reagents as described previously (Timsina & Hewick 1990). Plasma samples were diluted either 1 in 40 (samples from 5 min to 2 h blood collection period) or 1 in 20 (from 3 to 7 h blood collection period). All determinations were carried out in triplicate.

Determination of extracellular fluid volume. The thiocyanate space, used as an index of extracellular fluid volume (ECFV), was determined as described previously (Timsina & Hewick 1990).

Pharmacokinetic and statistical analysis. The DSFab plasma concentration vs time data were analysed as follows. The elimination rate constant (k_{el}) and elimination half-life ($t_{\frac{1}{2}\beta}$) were obtained by a model-independent method from the terminal part (3 to 7 h) of the log concentration vs time plot using linear least-squares regression analysis. The total area under the concentration vs time curve (AUC) was obtained from 0 to 7 h using the linear trapezoidal rule and from 7 h to infinity using k_{el} to determine the extrapolated area.

The total digoxin plasma concentration vs time data either for the control experiment or after DSFab administration were analysed using the above method. The k_{el} and total [^{3}H]digoxin $t_{\frac{1}{2}\beta}$ were obtained from the terminal part (24 to 103·5 h) of the log concentration vs time plot. The AUC was obtained from 0 to 103·5 h using the linear trapezoidal rule and from 103·5 h to infinity by extrapolation using k_{el}.

Using the parameters obtained, the apparent volume of distribution at steady state (V_{dss}) was calculated by dividing the product of dose and area under the moment curve (AUMC) by $(AUC)^2$ (Gibaldi & Perrier 1982). The AUMC (the area under the curve of the product of time and plasma concentration over the time-span zero to infinity) was calculated in the same way as AUC (see above). Systemic clearance was calculated by dividing the dose by AUC.

To calculate digoxin systemic clearance and apparent volume of distribution after DSFab administration, it is necessary to estimate the amount of drug in the body just before DSFab dosing. This can be done using data obtained from the control digoxin experiment (method as described by Owens & Mayersohn (1986)). However, calculations correcting for the amount of digoxin dose eliminated in the hour before DSFab injection, showed only a negligible loss (1–2% of the administered digoxin dose). This is presumably because digoxin has to distribute into a large volume, and also because it has a relatively long $t_{\frac{1}{2}\beta}$ (see Results).

To obtain the distribution half-life ($t_{\frac{1}{2}\alpha}$) for DSFab, and digoxin in the control experiment when no DSFab was given, an exponential stripping programme (Brown & Manno 1978) was used.

To test for significant differences ($P < 0·05$) Student's paired *t*-test was used.

Results

The effect of DSFab on plasma digoxin disposition for the full 5 day experimental period is illustrated in Fig. 1. Greater detail for the initial 8 h, along with DSFab concentration is shown in Fig. 2. Fig. 1 indicates that the elevation of total digoxin concentration, caused by DSFab binding to digoxin in the plasma, was still apparent at 100 h ($P < 0·05$), even though the antibody fragments were not detected by 24 h (concentrations $< 0·05$ μg mL^{-1}). After an initial marked oscillation in free digoxin concentration this, unlike total digoxin concentration, remained at a relatively constant low level during the 7 to 90 h period. The influence of DSFab on elimination-related pharmacokinetic parameters of digoxin is shown in Table 1. The $t_{\frac{1}{2}\beta}$, V_{dss} and clearance values were reduced by some 40, 90 and 75% respectively. The $t_{\frac{1}{2}\alpha}$ for digoxin in the control (no DSFab) experiment was $0·17 \pm 0·02$ h.

The initial effects of DSFab are more readily seen in Fig. 2. The well-established (4- to 5-fold) rise in total digoxin was accompanied by a two-thirds fall in free digoxin concentration within 15 min of DSFab administration. However, subsequently there was a consistent rise, so that by 60 min the net reduction in

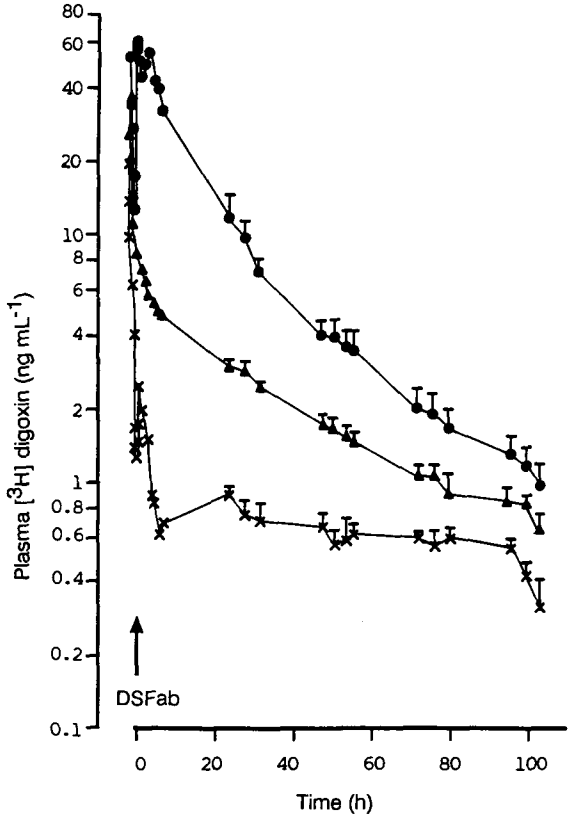

FIG. 1. The effect of DSFab administration on total (●) and free (×) digoxin plasma concentrations. ▲ Total digoxin concentrations in a control experiment performed 4 weeks earlier or later and in which no DSFab were given. The doses of digoxin and DSFab were 15 μg kg^{-1} and 1·9 mg kg^{-1}, respectively. The DSFab given at 0 h was administered 1 h after the digoxin. The data points are mean ± s.e.m. from five rabbits. Note that s.e.m. for the initial 8 h are not shown.

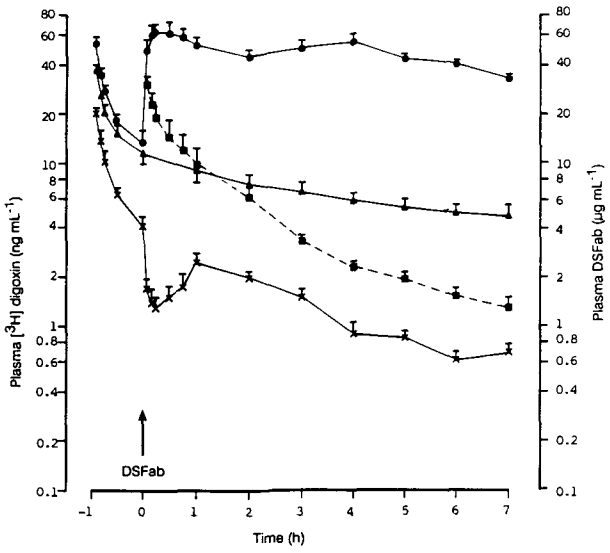

FIG. 2. Plasma concentration vs time profile for total and free digoxin during the initial period of the study depicted in Fig. 1. The symbols for total and free digoxin are as in Fig. 1, while DSFab are depicted by solid squares/broken line. Means ± s.e.m. are given (n = 5).

Table 1. The effect of DSFab administration on digoxin plasma elimination kinetics in rabbits.

Parameter	Control	DSFab administration
$t_{\frac{1}{2}\beta}$ (h)	37·7 ± 2·9	22·7 ± 1·2*
V_{dss} (L kg^{-1})	2·8 ± 0·2	0·3 ± 0·04*
CL (mL kg^{-1} h^{-1})	57·1 ± 3·9	14·0 ± 1·6*

The i.v. doses of [^{3}H]digoxin and DSFab were 15 μg kg^{-1} (19 μmol kg^{-1}) and 1·9 mg kg^{-1} (38 μmol kg^{-1}), respectively. Means ± s.e.m. are given (n = 5). * $P < 0·05$, paired t-test.

free digoxin concentration was only one-third. During this initial period, the pharmacokinetic parameters for DSFab were $t_{\frac{1}{2}\alpha}$, 0·3 ± 0·1 h; $t_{\frac{1}{2}\beta}$, 3·2 ± 0·6 h; V_{dss}, 184·7 ± 18·0 mL kg^{-1} and clearance 56·9 ± 2·1 mL kg^{-1} h^{-1}.

The thiocyanate space, used as an index of ECFV, in the five rabbits used was 198 ± 12 mL kg^{-1}.

Table 2 gives information on the stoichiometry between digoxin and DSFab during the first 120 min. The rebound rise in free digoxin concentration after 15 min occurred in all five rabbits and became significant at 45 min, reaching a maximum at 60 min. The minimum concentration of free digoxin was maintained with a DSFab:digoxin molar concentration ratio of 4·8 but values of 3·7 or lower were associated with an increase in free digoxin concentration. By 7 h the molar concentration ratio had fallen to 0·6.

Discussion

The increase in total plasma digoxin concentration after digoxin followed by DSFab administration is well documented both in animals (Schmidt et al 1971; Butler et al 1977; Ochs et al 1978; Hewick et al 1986; Johnston et al 1987) and man (Smith et al 1976; Rozkovec & Coltart 1982; Smolarz et al 1985; Sinclair et al 1989). However, in the current studies, the prolonged evaluation in plasma digoxin is surprising in view of the short (3 h) elimination half-life of DSFab in the rabbit, resulting in 24 h antibody plasma concentrations of less than 0·05 μg mL^{-1} and DSFab:total digoxin molar concentration ratio of below 0·06. It seems that further distribution and elimination, after the initial DSFab-induced redistribution of digoxin is slower than would be anticipated. Although the $t_{\frac{1}{2}\beta}$ total digoxin was reduced by DSFab, the decrease in V_{dss} indicated by the increase in AUC, produced a significant net decrease in systemic clearance (dose/AUC). This effect of drug-specific Fab fragments has also been shown for the clearance of phencyclidine in dogs (Owens & Mayersohn 1986). However, the reduction in systemic drug clearance was apparently not detectable in terms of urinary excretion rates for either digoxin (Butler et al 1977) or phencyclidine (Owens & Mayersohn 1986).

In addition to the present study, the rebound in free digoxin concentration occurring shortly after drug-specific Fab administration has been noted in man (Smith et al 1976; Schaumann et al 1986; Hursting et al 1987; Sinclair et al 1989), for phencyclidine in dogs (Owens & Mayersohn 1986) and desipramine in rabbits (Hursting et al 1989). In many of these previous studies at least an equimolar dose of Fab fragments had been given with respect to the hapten. In the current study, the finding that a twice-molar DSFab dose did not prevent the rebound indicates that giving a higher initial antibody dose may not be a suitable way of eliminating the phenomenon. This is supported by the observation that a similar magnitude of rebound was observed whether twice-molar or equimolar (preliminary experiments on two rabbits, data not shown) DSFab were used. It has been suggested (Owens & Mayersohn 1986) that the rebound is partly an experimental artifact; drug-specific Fab fragments may degrade (presumably in-vivo) into smaller drug-binding molecules which

Table 2. Plasma digoxin and DSFab concentrations during a 120 min period after DSFab administration.

Time after DSFab (min)	Digoxin (ng mL^{-1})		DSFab (μg mL^{-1})	Digoxin bound (%)	Molar concn ratio (DSFab: total digoxin)
	Total	Free			
−2	13·3 ± 2·3	4·10 ± 0·6	0·0	30·8	0·0
5	48·4 ± 7·0	1·73 ± 0·2	30·1 ± 4·2	96·4	9·7
10	60·2 ± 9·0	1·43 ± 0·3	23·1 ± 4·2	97·6	6·0
15	62·1 ± 7·9	1·33 ± 0·2	19·1 ± 4·0	97·9	4·8
30	61·9 ± 8·4	1·47 ± 0·3	14·4 ± 4·2	97·6	3·7
45	58·4 ± 6·7	1·76 ± 0·3*	11·9 ± 3·4	97·0	3·2
60	52·6 ± 5·7	2·50 ± 0·3*	9·8 ± 2·6	95·5	2·9
120	45·1 ± 3·7	2·01 ± 0·2*	6·1 ± 1·1	95·2	2·1

Means ± s.e.m. are given (n = 5). • $P < 0·05$, paired t-test compared with the minimum free digoxin concentration at 15 min.

can pass through the semi-permeable membranes used in the determination of the free drug concentrations. They reported (using serum samples from two dogs) that when the pore size of the membrane was reduced (from about 13 000 daltons) so that it became impermeable to molecules of mol. wt greater than 3–5000 daltons, the rebound effect was abolished. However, we have found that there is still a rebound when a membrane with a 5000 dalton mol. wt cut-off is used. An alternative explanation for the phenomenon is that a significant portion of uncomplexed DSFab is cleared from the plasma allowing free digoxin to re-accumulate, due to continued diffusion of the drug from the tissues.

In the present study total plasma radioactivity was measured, which may contain metabolites along with unchanged digoxin, and it is conceivable that their presence could provide an explanation for the rebound effect. However, a rebound effect still occurs in man in which digoxin is only minimally metabolized (Gault et al 1984).

Our studies agree with those of Sinclair et al (1989) in that a molar DSFab:total digoxin ratio of about 5 is necessary to maintain minimum free digoxin concentrations, and where the ratio is less than four during the initial treatment period, free concentrations will rise. It was suggested by Sinclair et al (1989) that the DSFab:total digoxin ratios as calculated could be misleading because the DSFab assay being used measures sheep protein and not digoxin binding capacity on which the antidotal effect depends. However, subsequent discussions with the manufacturer of Digibind (Dr G. Allen, Head, Department of Protein Chemistry, Wellcome Foundation Ltd) elicited the information that the expected binding capacity of Digibind (molecules of digoxin bound per molecule of DSFab expressed as a percentage) was about 97%. Thus it seems that within the plasma, a considerable molar excess of DSFab is required to minimize free digoxin concentrations.

In conclusion, although we have indicated apparent problems such as a reduction in digoxin clearance and an adverse oscillation in free digoxin concentration, it must be reaffirmed that in the clinical situation the general effectiveness of DSFab in reversing acute digoxin toxicity is not in doubt.

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