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The Use of Enzyme-linked Immunosorbent Assays to Study the Plasma Disposition of Sheep Polyclonal and Rat Monoclonal Digoxin-specific Fab Fragments in the Rabbit

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Abstract—The plasma disposition of sheep polyclonal digoxin-specific Fab (fragment antigen-binding) fragments has been studied in rabbits after their intravenous injection (1 mg kg^{-1}) using enzyme-linked immunosorbent assays exploiting both the species-specificity ($ELISA_1$) and the digoxin-specificity ($ELISA_2$) of digoxin-specific Fab fragments. The log concentration versus time profiles were best described by a biexponential plasma disposition when either assay was used. Although the plasma concentrations determined by $ELISA_1$ and $ELISA_2$ at each sampling time were not significantly different, there was a tendency for certain $ELISA_2$ values to be higher. This resulted in the $ELISA_2$-derived data giving a significantly longer distribution half-life ($t_{\frac{1}{2}}\alpha$), but similar values for elimination half-life ($t_{\frac{1}{2}}\beta$), apparent volume of distribution at steady state (Vd_{ss}), and clearance. Using $ELISA_2$, which was generally the more sensitive assay, to compare the plasma disposition of the sheep polyclonal digoxin-specific Fab fragments with rat monoclonal digoxin-specific Fab fragments, it was shown that the rat product had a shorter $t_{\frac{1}{2}}\alpha$ (11 vs 22 min), a $t_{\frac{1}{2}}\beta$ which was not significantly different (253 vs 168 min), but a faster clearance (1·2 vs 0·7 mL kg^{-1} min^{-1}), associated with a much larger Vd_{ss} (321 vs 108 mL kg^{-1}). The extracellular fluid volume, using thiocyanate as a marker, was about 216 mL kg^{-1} for the nine rabbits used. This suggests that the rat preparation penetrates more extensively into the extracellular space and may indicate that some degree of extracellular binding or cell penetration is occurring.

Digoxin-specific Fab (fragment antigen binding) fragments, derived from sheep immunoglobulin G (IgG), are used to treat severe cardiac glycoside intoxication (Wenger et al 1985). They may also have a use as a diagnostic aid in suspected cardiac glycoside overdose involving dysrhythmias (Bagrov et al 1989) and in the treatment of pathological conditions such as eclampsia (Goodlin 1988, 1989) where 'endogenous digitalis' is postulated to have a role. Furthermore, drug-specific Fab fragments could have a use in the treatment of poisoning by other drugs and toxins such as phencyclidine (Owens & Mayersohn 1986), desipramine (Hursting et al 1989) and tetrodotoxin (Huot et al 1989).

Although digoxin-specific Fab fragments used clinically are derived from polyclonal antibodies raised in sheep, there is an increasing tendency to prepare rodent monoclonal antibodies which can be used in drug toxicity reversal (Mudgett-Hunter et al 1982; Bowles et al 1988; Huot et al 1989). Indeed, it has been demonstrated that mouse monoclonal digoxin-specific Fab fragments effectively reversed the deleterious effects of a potentially lethal dose of digoxin in guinea-pigs (Lechat et al 1984). With the possibility of an increased use of rodent monoclonal drug-specific Fab fragments in mind, we carried out preliminary experiments in the rabbit in which the plasma disposition of sheep polyclonal versus rat monoclonal digoxin-specific Fab fragments was compared. It was found that with the same injected dose of digoxin-specific Fab fragments, the rat monoclonal Fab preparation gave much lower plasma concentrations. Unfortunately the ELISA procedure employed (based on the species-specificity of digoxin-specific Fab fragments), which we have previously used to study sheep polyclonal digoxin-specific Fab fragments disposition in laboratory animals and man (Johnston et al 1988; Sinclair et al 1989; Timsina et al 1989), had insufficient sensitivity to measure reliably the low monoclonal digoxin-specific Fab plasma concentrations. Therefore in the present investigation we have produced and tested a modified ELISA (based on the digoxin-specificity of digoxin-specific Fab fragments), which preliminary experiments have shown to have a greater sensitivity, and then used this assay to compare the plasma disposition of rat monoclonal with sheep polyclonal digoxin-specific Fab preparations.

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Materials and Methods

We received as gifts from the Wellcome Foundation Ltd., two digoxin-specific Fab preparations: the commercial product (Digibind) derived from sheep polyclonal IgG, and an experimental compound derived from rat monoclonal IgG_1. Donkey anti-sheep IgG, sheep anti-rat gammaglobulin, donkey serum, and sheep serum were supplied by the Scottish Antibody Production Unit, Carluke, UK. Anti-sheep IgG alkaline phosphatase conjugate (donkey, 515 enzyme units mL^{-1}), anti-rat IgG alkaline phosphatase conjugate (rabbit, 590 enzyme units mL^{-1}), *p*-nitrophenyl phosphate, human serum albumin (fraction V) and digoxin were purchased from Sigma Chemical Co., Poole, UK. Polystyrene microtitre plates (96 flat-bottom wells, 0·4 mL capacity) were obtained from Gibco, Paisley, UK. All other reagents were obtained from British Drug Houses, Poole, UK and were of analytical grade. Rabbit plasma was collected from individual untreated rabbits in the laboratory.

Preparation of digoxin-human serum albumin conjugate

A digoxin-human serum albumin (HSA) conjugate was prepared by the method of Smith et al (1970) in which the terminal digitoxose residue of digoxin is oxidized with sodium metaperiodate so that the resulting dialdehyde reacts with a primary amino group of the albumin to form a Schiff base linkage. The bond is then stabilized by reduction with sodium borohydride. Using the spectrophotometric method of Smith et al (1970), it was estimated that the conjugate possessed 10 digoxin molecules per molecule of albumin.

Enzyme-linked immunosorbent assays for sheep or rat digoxin-specific Fab

Species-specific ($ELISA_1$) and digoxin-specific ($ELISA_2$) assays were used. For $ELISA_1$, wells of microtitre plates were coated with 0·25 mL of anti-sheep IgG (1:1000), or anti-rat gammaglobulin (1:500) in distilled water. For $ELISA_2$, digoxin-HSA conjugate 0·25 mL (0·2 mg mL^{-1} in bicarbonate buffer, 0·05 M, pH 9·6) was used for coating.

The plates were incubated for 30 min at 37 °C and then overnight at 4 °C. The following day, plates were washed three times with phosphate buffered saline (0·01 M, pH 7·4) containing 0·05% Tween-20 (PBS-Tween). The assay then proceeded as follows. Plasma samples for $ELISA_1$ were diluted 1:20 (samples from 5–120 min blood collection period) or 1:10 (from 150–360 min blood collection period) and for $ELISA_2$ the samples were diluted 1:40 or 1:20, respectively (diluent PBS-Tween). The diluted sample (0·2 mL) containing sheep polyclonal or rat monoclonal digoxin-specific Fab fragments was added to each well and incubated for 1 h at 37 °C. The plates were washed three times with PBS-Tween. Donkey anti-sheep or rabbit anti-rat IgG alkaline phosphatase conjugate (0·2 mL 1:1000 in PBS-Tween containing 5% donkey or sheep serum with 2·5% rabbit plasma) was added to each well and incubation carried out for 1 h at 37 °C. The plates were washed three times with PBS-Tween, then 0·2 mL of substrate (*p*-nitrophenyl phosphate, 2 mg mL^{-1} in substrate buffer, pH 10·4) was added to each well and incubated for 30 min at 25 °C. Finally, 0·05 mL of NaOH (1 M) was added to each well to stabilize the colour reaction. The absorbances were read at 405 nm using an automatic plate reader (Dynatech, MR 580). Standard calibration curves of sheep polyclonal or rat monoclonal digoxin-specific Fab fragments were constructed over the range 0·05 to 1 $\mu g\ mL^{-1}$ for $ELISA_1$ and 0·1 to 0·5 $\mu g\ mL^{-1}$ for $ELISA_2$. The standard solutions contained the same amount of rabbit plasma as the test dilutions.

Procedure in conscious rabbits

Female New Zealand-White rabbits (3·6–4·6 kg) were used. The experimental procedure was carried out in conscious animals and digoxin-specific Fab fragments (1 mg kg^{-1}, 1 mg mL^{-1} saline) were injected into the marginal vein of the right ear of each animal. Blood was collected (nominally at 5, 10, 15, 30 min and then every 30 min until 360 min) via a cannula from the marginal vein of the left ear. The plasma was obtained by centrifuging blood samples for 15 min at 3000 *g* and the samples were stored at −20 °C as aliquots until analysed.

Rat monoclonal and sheep polyclonal digoxin-specific Fab preparations were compared separately in the same rabbits with a period of about three weeks between the two administrations. In half the rabbits, the rat preparation was given first, while the sheep preparation was given first in the other half.

Determination of extracellular fluid volume

The thiocyanate space was used as an index of extracellular fluid volume (ECFV), employing a method derived from that of Bianchi et al (1981) for rats. Plasma samples (0·1 mL), taken at 180 min after i.v. injection of 100 mg sodium thiocyanate (NaSCN), were added to 0·8 mL ice-cold trichloroacetic acid (12·5% w/v) followed by 1 mL distilled water. After 30 min and centrifugation (15 min, 3000 *g*), 0·8 mL of supernatant was added to 0·8 mL of ferric nitrate (0·12 M) and the absorbance of the resultant amber-coloured ferric thiocyanate solution was determined at 480 nm using a Pye-Unicam SP-500 spectrophotometer. A standard ferric thiocyanate solution was prepared by adding 0·1 mL of NaSCN (1 mg mL^{-1}) to 0·8 mL ice-cold trichloroacetic acid (12·5% w/v) and carrying out the subsequent procedures as described above. All samples and standards were prepared in triplicate. The ECFV (mL kg^{-1}) for each animal was calculated by dividing the mean absorbance of the standard by that of the sample, dividing by the weight (kg) of the rabbit, and multiplying by 100.

Pharmacokinetic and statistical analysis

The digoxin-specific Fab plasma concentration versus time data were analysed as follows. The elimination rate constant (K_{el}) and elimination half-life ($t_{\frac{1}{2}}\beta$) were obtained from the terminal part (210–360 min) of the log concentration versus time plot using least-squares regression analysis. The area under the concentration versus time curve (AUC) was obtained from 0–210 min by the linear trapezoidal rule and from 210 min to infinity by extrapolation using K_{el}. Using the parameters obtained, the apparent volume of distribution at steady state (Vd_{ss}) was calculated by dividing the product of dose and area under the moment curve by $(AUC)^2$ (Gibaldi & Perrier 1982). Clearance was calculated by dividing the dose by AUC. To obtain information on the distribution phase and to obtain the distribution half-life ($t_{\frac{1}{2}}\alpha$), an exponential stripping programme (Brown & Manno 1978) was used.

The F-test ($P < 0·05$) was used to test which compartmental model best fitted the plasma concentration versus time profile.

Significant differences were detected using the paired *t*-test with $P < 0·05$ being taken as significant.

Results

Fig. 1 compares $ELISA_1$ and $ELISA_2$ in the production of calibration curves for sheep polyclonal (Fig. 1a) and rat monoclonal (Fig. 1b) digoxin-specific Fab fragments. It is seen that for the respective assays, antibody fragments from the two species produce similar calibration curves. However, for both species, the hapten-specific assay ($ELISA_2$) appears more satisfactory, in that in general the sensitivity is greater and a linear absorbance versus concentration relationship is obtained over a suitable Fab concentration range.

Fig. 2 compares the plasma elimination of sheep Fab

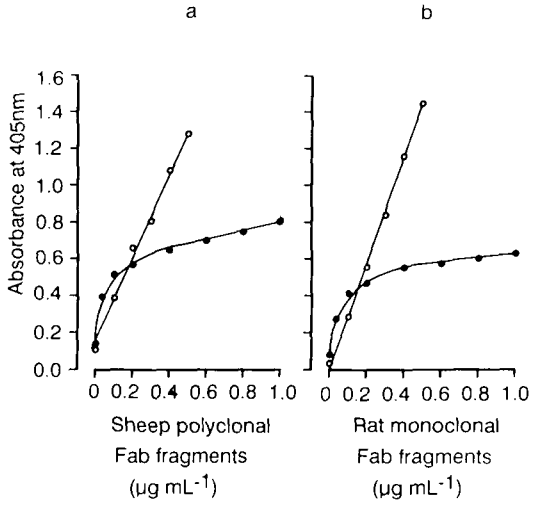

FIG. 1. Calibration curves for species-specific ($ELISA_1$, closed circles) and hapten specific ($ELISA_2$, open circles) immunoassays in the measurement of sheep polyclonal (a) or rat monoclonal (b) digoxin-specific Fab fragments. The points are means of triplicates.

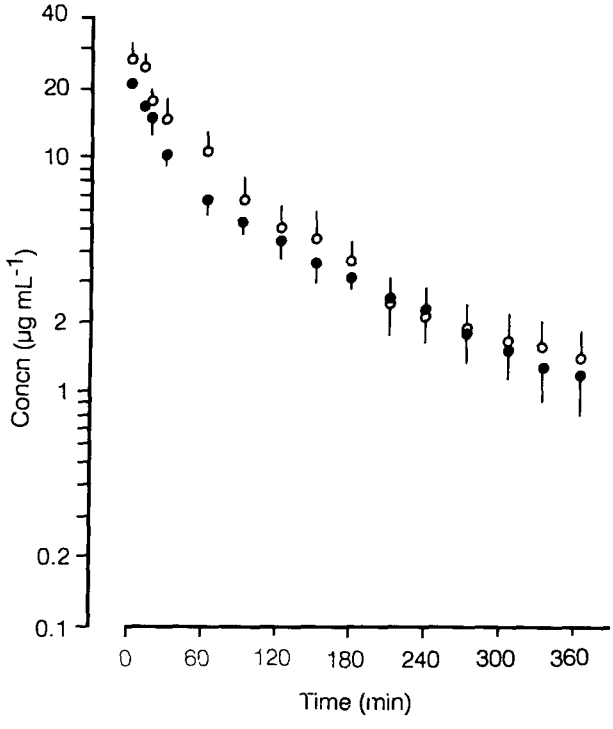

FIG. 2. The plasma elimination of sheep digoxin-specific Fab fragments in the rabbit, as measured using species-specific ($ELISA_1$, closed circles) or hapten-specific ($ELISA_2$, open circles) immunoassays. The dose of digoxin-specific Fab fragments was 1 mg kg^{-1} i.v. Means ± s.e.m. are given (n = 5).

fragments in the rabbit, using $ELISA_1$ or $ELISA_2$ to measure the antibody concentrations. Both assays appeared to give a generally similar picture of biexponential elimination, and there were no significant differences (paired t-test, $P < 0.05$) in Fab concentration determined by $ELISA_1$ or $ELISA_2$. However, there appeared to be a tendency for $ELISA_2$ to produce higher concentration values particularly in the first half and the latter part of the 6 h experimental period. This produced one significant difference in the pharmacokinetic parameters calculated from the two sets of data. The $t_{\frac{1}{2}}\alpha$ obtained using $ELISA_2$ was longer ($P < 0.05$) but the pairs of $t_{\frac{1}{2}}\beta$, Vd_{ss} and clearance values were similar (Table 1). The mean thiocyanate space for the five rabbits used was 215·5 ± 14·4 mL kg^{-1}.

The more sensitive hapten-specific assay ($ELISA_2$) was used to compare the elimination of rat versus sheep preparations. Fig. 3 shows that, after injection of identical amounts of antibody preparation, lower plasma concentrations of the rat product were obtained. The antibody preparations from both species exhibited biexponential plasma kinetics. The corresponding pharmacokinetic parameters obtained for the four rabbits are given in Table 2. The shorter $t_{\frac{1}{2}}\alpha$ value, three-fold greater volume of distribution and increased clearance for the rat monoclonal Fab fragments were significantly different from the corresponding parameters for the sheep preparation.

The thiocyanate space for the four rabbits was 215·8 ± 8·6 mL kg^{-1}.

Table 1. Pharmacokinetic parameters for sheep polyclonal digoxin-specific Fab fragments in rabbits using $ELISA_1$ and $ELISA_2$.

Parameter	$ELISA_1$	$ELISA_2$
$t_{\frac{1}{2}}\alpha$ (min)	9·6 ± 2·3	31·6 ± 6·7*
$t_{\frac{1}{2}}\beta$ (min)	146·4 ± 34·0	186·0 ± 24·0
Vd_{ss} (mL kg^{-1})	105·2 ± 8·1	105·5 ± 23·6
Clearance (mL kg^{-1} min^{-1})	0·62 ± 0·09	0·58 ± 0·15

* $P < 0.05$. All values mean ± s.e.m. (n = 5).

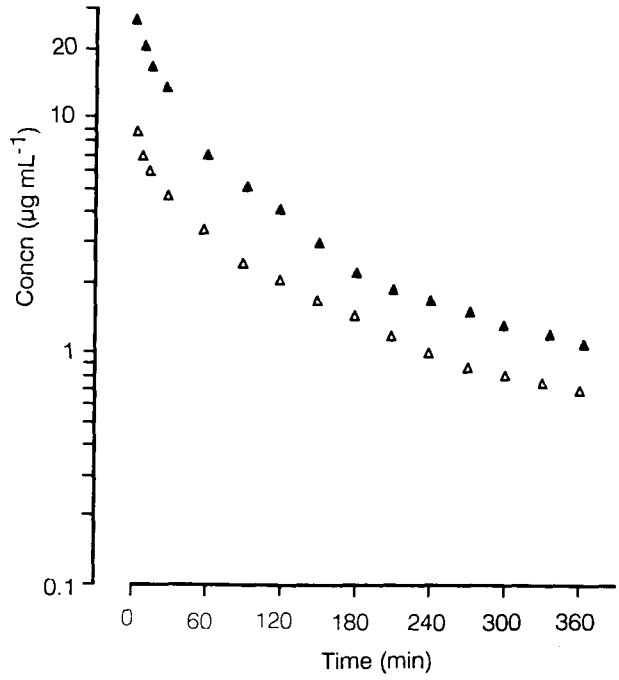

FIG. 3. The plasma elimination of sheep polyclonal (closed triangles) or rat monoclonal (open triangles) digoxin-specific Fab fragments in the rabbit as measured using a hapten-specific ($ELISA_2$) immunoassay. The dose of digoxin-specific Fab fragments was 1 mg kg^{-1} i.v. As blood sampling times varied slightly from rabbit to rabbit, data from one rabbit only are presented to clarify presentation. The points are means of triplicate assays.

Table 2. Pharmacokinetic parameters for sheep polyclonal versus rat monoclonal digoxin-specific Fab fragments in rabbits using $ELISA_2$.

Parameter	Sheep polyclonal	Rat monoclonal
$t_{\frac{1}{2}}\alpha$ (min)	22·2 ± 7·0	11·3 ± 4·2*
$t_{\frac{1}{2}}\beta$ (min)	167·8 ± 17·6	252·6 ± 58·2
Vd_{ss} (mL kg^{-1})	107·7 ± 7·4	320·9 ± 53·3*
Clearance (mL kg^{-1} min^{-1})	0·74 ± 0·11	1·17 ± 0·21*

* $P < 0·05$. All values mean ± s.e.m. (n = 4).

Discussion

The hapten-specific immunoassay ($ELISA_2$) gave a more satisfactory calibration curve than the species-specific assay ($ELISA_1$). Presumably the reduced sensitivity of $ELISA_1$ was due to a lower specific binding activity of the anti-species coating protein which, in consequence, became more readily saturated with Fab fragments. However, it should be mentioned that despite the unflattering comparison of $ELISA_1$ with $ELISA_2$ in Fig. 1, the former assay can be used effectively to measure sheep polyclonal digoxin-specific Fab plasma concentrations after clinical doses of this preparation (Sinclair et al 1989). $ELISA_2$ works well in the rabbit experimental model described in this paper, but suffers from a major disadvantage in that free hapten will interfere with the measurement of the drug-specific antibody fragments. We have found (unpublished data) that an equimolar concentration of digoxin reduces by about 80% the absorbance values produced by $ELISA_2$ in the measurement of digoxin-specific Fab fragments. Therefore, $ELISA_2$ would be unsuitable for measuring digoxin-specific Fab fragment concentrations in patients being treated for digoxin overdose.

Comparison of $ELISA_1$ and $ELISA_2$ for monitoring the plasma elimination of sheep polyclonal digoxin-specific Fab fragments suggests that, although there was no significant difference between the two sets of values obtained at each sampling time, there was a tendency for $ELISA_2$ to produce higher Fab concentration values during the first 180 min and the last 60 min of the 6 h experimental period. This resulted in $ELISA_2$-derived data producing a significantly longer distribution half-life, although the elimination half-life the Vd_{ss} values and clearances derived from the two sets of assay results were similar. The significance of the discrepancy is unclear. It should be noted that the plasma concentration data for the rabbit as obtained in the present study appear to better fit a biexponential rather than a triexponential profile reported previously (Timsina et al 1989).

The Vd_{ss} values obtained for sheep polyclonal digoxin-specific Fab fragments in the present experiment (105–108 mL kg^{-1}) were in good agreement with those (about 115 mL kg^{-1}) previously reported (Timsina et al 1989). There was also good agreement between the values obtained for the thiocyanate space (216 vs 220 mL kg^{-1}).

The three-fold greater Vd_{ss} value calculated for rat monoclonal digoxin-specific Fab fragments was surprising. If it is assumed that the thiocyanate space approximates to the ECFV, the Vd_{ss} values for rat monoclonal and sheep polyclonal digoxin-specific Fab fragments of 321 and 108 mL kg^{-1}, respectively, suggest that the rat preparation penetrates the extracellular space more extensively. The value for the volume of distribution of rat monoclonal digoxin-specific Fab fragments in the rabbit (321 mL kg^{-1}) is similar to those reported for sheep polyclonal digoxin-specific Fab in the baboon (280–461 mL kg^{-1} Smith et al 1979) and man (322 mL kg^{-1} Sinclair et al 1989). It seems that sheep polyclonal digoxin-specific Fab fragments distribute less readily in smaller animals (Timsina et al 1989), while with the rat monoclonal digoxin-specific Fab preparation, this interspecies variation in distribution may not occur. The fact that the Vd_{ss} value for rat monoclonal digoxin-specific Fab fragments appears to exceed the rabbit ECFV could suggest some degree of extracellular binding or cell penetration. With the likelihood of rat or mouse monoclonal antibody preparations being more widely used, the reported difference in the extent of distribution is of interest. Possible reasons for the difference include differences in species in which the antibodies were raised, the sub-class of IgG (from which the Fab fragments were derived) and inherent dissimilarities between monoclonal and polyclonal Fab fragments.

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