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A Radioimmunoassay Technique for Digoxin in Postmortem Blood

In spite of the availability of purified digitalis fractions such as digoxin, treatment of congestive heart failure and arrhythmias with cardiac glycosides is attended by considerable risk of toxicity and even death. Beller et al [*1*] estimate that toxicity results in 8-20 percent of hospital patients taking digoxin, with a subsequent mortality of 7-50 percent. The development of a radioimmunoassay sufficiently sensitive to quantitate digoxin in serum or plasma during therapy [*2*] might be expected to improve the control of therapy in patients with absorption or excretion abnormalities, and Smith and Haber [*3*] reported that more than 85 percent of toxic cases could be distinguished from nontoxic ones by the plasma digoxin concentrations. However, more recently, Fogelman et al [*4*] found little increase in plasma digoxin in toxicity; mean plasma concentrations were 1.69 ± 1.29, 1.61 ± 0.79, and 1.41 ± 1.09 ng/ml in groups classified as toxic, possibly toxic, and nontoxic. The extensive overlap of the digoxin concentrations in these three groups suggests that sensitivity to digoxin is the main factor in determining the onset of toxicity.

Apart from the fatalities which are expected to result from a therapy which is so poorly understood, cases involving massive digoxin overdosage, due to accident or suicide, are sometimes encountered in forensic science. Assay methods based on ethanol extraction [*5*] and direct thin-layer chromatography (TLC) [*6*] had been used in the forensic science laboratory, but with minimum detection limits around 50 μg, the methods were quite inadequate for following the primary response to digoxin overdose, elevation of blood concentrations. As described herein the plasma digoxin radioimmunoassay has been adapted for use with postmortem blood. A preliminary report of the method has been published [*7*].

The principle of the unmodified assay is that the reaction of a limiting quantity of antidigoxin serum with a quantity of ^{3}H-dixogin is inhibited by the digoxin contributed by a test sample. The degree of inhibition may be related to the performance of a series of known digoxin solutions expressed as a standard curve. ^{3}H-digoxin not bound to antibody is adsorbed onto charcoal and the ^{3}H-digoxin-antibody complex in the charcoal-free supernatant is counted in a liquid scintillation spectrometer.

When plasma or serum at 10 percent in the final reaction mixture (including charcoal) was replaced by postmortem blood, the severe color quenching of the scintillation process caused by heme could be somewhat alleviated by decolorization with a hypochlorite bleach [*8,9*]. However, count rates were typically no better than a third those of serum-

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containing samples, and both the sensitivity and reproducibility were greatly diminished. After considerable digoxin overdosage postmortem blood often contains so much digoxin that direct assay of the blood at only 1-2 percent in the reaction mixture is feasible, and at this dilution heme effects are small. However, to allow universal application, the technique was modified so that hemoglobin is absent from the solution submitted to scintillation counting. This was achieved by extraction of digoxin from the postmortem blood by dialysis against the antiserum. The dialysis method, in common with the novel method for serum digoxin radioimmunoassay [*10*], exploits differences in the binding kinetics of various drugs with the antibody, and as a consequence it is highly sensitive and specific.

Materials

Aldadiene and spironolactone were donated by G. D. Searle and Co., prednisone by Roussel Laboratories Ltd., and prednisolone by Boots Pure Drug Co. Digitoxin was purchased from Sigma Chemical Co. Stock solutions of these steroids were made at 1 mg/ml in ethanol.

Digoxin, tritiated digoxin, antidigoxin serum, normal horse serum (NHS), charcoal suspension, and a phosphate buffer were obtained from the Lanoxitest B digoxin radioimmunnoassay kit of Wellcome Reagents Ltd. At its working strength the buffer contained 0.5 percent bovine serum albumin (BSA) and 0.1 percent sodium azide in 0.04 *M* sodium phosphate pH 7.4. Digoxin in the kit was Wellcome Lanoxin® dissolved at 100 ng/ml in the buffer. Radioactive digoxin was digoxin-12*a*-^{3}H, specific activity at least 6 C/mM, in solution at 100 ng/ml in buffer. The charcoal suspension was comprised of 25 g Norit A charcoal in 0.12 *M* barbitone buffer pH 8.6 containing 0.2 percent BSA and 0.1 percent sodium azide. Latterly, a suspension of Norit A charcoal (Norit Clydesdale Ltd.) in the phosphate buffer was used. The rabbit antiserum to digoxin-albumin conjugate available initially was Lot K3864, superseded by Lot K4996, reputedly of better specificity. Each vial of lyophilized antiserum was reconstituted immediately before use with deionized water, 20 ml and 15 ml, respectively, for the successive antiserum batches, giving effective dilutions of 1/8000 and 1/18,000. Unused portions of antiserum were frozen for storage at -20°C, and thawed immediately prior to use. Other reagents were stored at 4°C. On occasion Wellcome No. 5 heated horse serum was used, 0.1 percent sodium azide being added to each bottle at the first use.

Dialysis were performed in ¼-in. Visking® tubing (The Scientific Instrument Centre Ltd.) held in GL5 polycarbonate tubes from Greywoods Laboratory Disposables Ltd. The rest of the radioimmunoassay was performed in disposable polythene tubes with captive tops, supplied for use with the Quickfit Instrumentation 320 Microcentrifuge (J. A. Jobling Ltd.) by Precision Machine Engineers (Harrow) Ltd.

The scintillator cocktail was comprised of 2/1 Analar toluene/scintillation grade triton X-100 (Intertechnique Ltd) containing 0.7 percent PPO and 0.035 percent POPOP (Koch Light Laboratories Ltd).

Radioimmunoassay Method

The standard digoxin solutions were made up in antidigoxin serum and left overnight at 4°C. Suitable digoxin standards were prepared by diluting the digoxin supplied by Wellcome Reagents Ltd. with antiserum to give 2 ng/ml, and serially diluting to give a total range of 2, 1.5, 1.0, 0.5, and 0 ng/ml in the antiserum.

Immediately prior to sampling, postmortem blood was ultrasonicated for about 10 s at 18,000 Hz in a Measuring and Scientific Equipment Ltd. Ultrasonicator®. Blood (or serum) was diluted with horse serum in order to obtain a digoxin concentration within

the standard curve. 0.4 ml of the sample was then dialyzed in Visking® tubing against the same volume of antiserum, overnight. GL5 plastic tubes, which had been individually preweighed, were used for the dialysis, being shaken overnight on a Luckham Ltd. Rotatest® operating at maximum speed in the 4°C cold room.

After 17-h dialysis, each dialysis sack was removed from its tube, the outside of the sack being washed down into the tube with phosphate buffer from a pasteur pipette during withdrawal. The dialysis sacks were discarded. The contents of each tube were then made up to 2.81 g with buffer.

The isotope dilution phase of the assay was performed at room temperature. Reaction mixtures, prepared at least in duplicate in the disposable microcentrifuge tubes, were of two types. The first, based on the dialyses, contained 0.7 ml of the diluted antiserum from the dialysis and 0.1 ml ^{3}H-digoxin at 5 ng/ml. The other series of reaction mixtures was comprised of 0.6 ml buffer, 0.1 ml ^{3}H-digoxin, and 0.1 ml antiserum either alone or containing standard digoxin. The tritiated digoxin was added last to each reaction mixture, and each tube was then allowed to stand for exactly 15 min. 0.2 ml charcoal suspension were then added in order to separate bound and free digoxin. Each tube was allowed to stand for 10 min with occasional shaking, before being centrifuged for 1.5 min in the Quickfit microcentrifuge (g max 14,000 xg fully loaded with 12 tubes). 0.7-ml supernatant portions were removed to high density polythene scintillation vials, and 0.4 ml of deionized water and 5.5 ml of the scintillator cocktail were added. Vials were counted for tritium in an Intertechnique SL31 liquid scintillation spectrometer with the autochanger chamber at 8°C, after equilibrating for at least 2 h. Samples were counted for 20 min, in which time approximately 270 counts accumulated per percent of ^{3}H-digoxin bound. Means of replicate results were taken.

The following controls were performed. A "total count" tube contained 0.1 ml of ^{3}H-digoxin 5 ng/ml solution plus 0.86 ml of buffer, which was not treated with charcoal but sampled directly for radioactivity counting. (0.96 ml is the total liquid volume in reaction mixtures containing charcoal, the volume of the solid charcoal being about 0.04 ml.) In order to verify the adsorbtion capacity of the charcoal, a "blank" tube was prepared containing 0.1 ml of ^{3}H-digoxin, 0.7 ml of buffer, and subsequently the 0.2 ml of charcoal suspension. Blank count rates were routinely about 30 counts/min. To guard against the remote possibility of radioactivity in postmortem blood [*11*], a sample radioactive blank was run comprised of 0.26 ml of buffer and 0.7 ml of the diluted antiserum from the blood dialysis; charcoal treatment was omitted.

On rare occasions, probably when the knot in the dialysis tubing was not sufficiently tight, traces of heme appeared outside the dialysis tubing. The presence of heme in such small amounts does not interfere with the reaction of digoxin with the antibody, but causes color quenching of the scintillation process. On such occasions all the scintillator vials were treated with a hypochlorite bleach before addition of the scintillant [*9*]. Taking this precaution where necessary, there was no evidence of any dissimilarity between the tritium counting efficiency in samples derived from standards or from post-mortem blood.

Where drug concentrations are stated, they relate to the blood, NHS, or antiserum before dilution during assay.

Results

Kinetics of Digoxin Extraction During Dialysis

A study of the kinetics of the digoxin extraction is shown in Fig. 1. Two nanograms per millilitre of ^{3}H-digoxin in normal horse serum were dialyzed against antiserum and

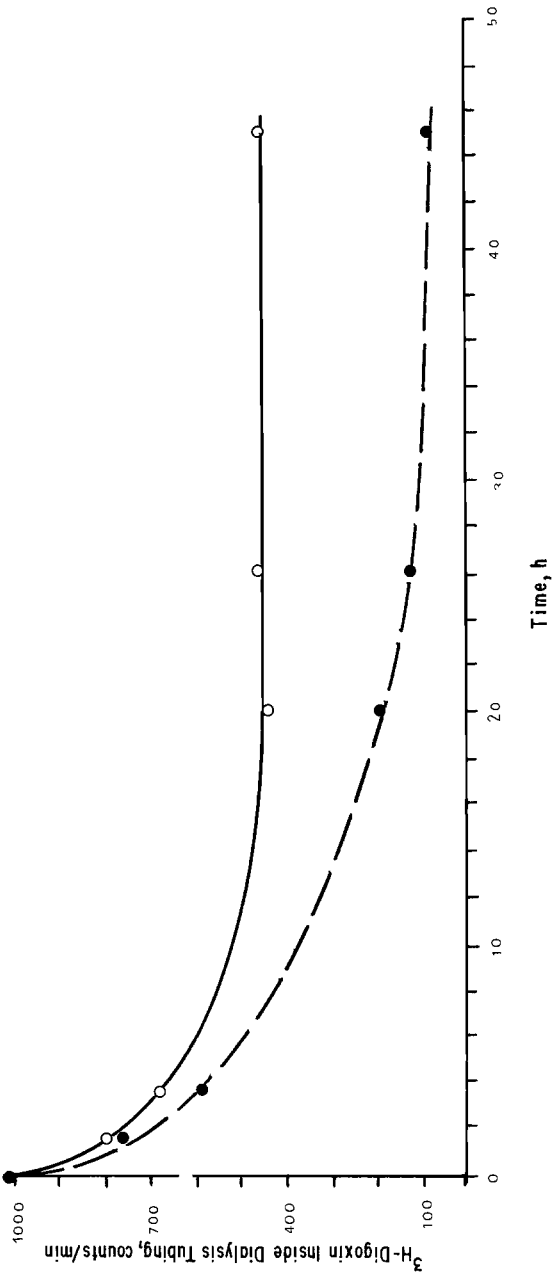

FIG. 1—*Kinetics of extraction of ^{3}H-digoxin from normal horse serum during dialysis against phosphate buffer* (open circles with solid line) *and against antiserum K3864* (solid circles with broken line).

against buffer, and the count rate of aliquots removed from within the dialysis sack is plotted against the time of sampling. A greatly reduced extraction rate results if the dialysis tubes are not shaken continuously.

Digoxin Standard Curves

A comparison was made of response curves prepared by the dialysis of digoxin standards in normal horse serum and in postmortem blood believed to be free of other digoxin. Response curves of five postmortem bloods were not significantly different from the serum-based standards, evidence of a similar extraction efficiency from the two media. A typical comparison of horse serum and postmortem blood standards may be seen in Fig. 2. The coefficient of variation in measurement at 1 ng/ml of digoxin was 12

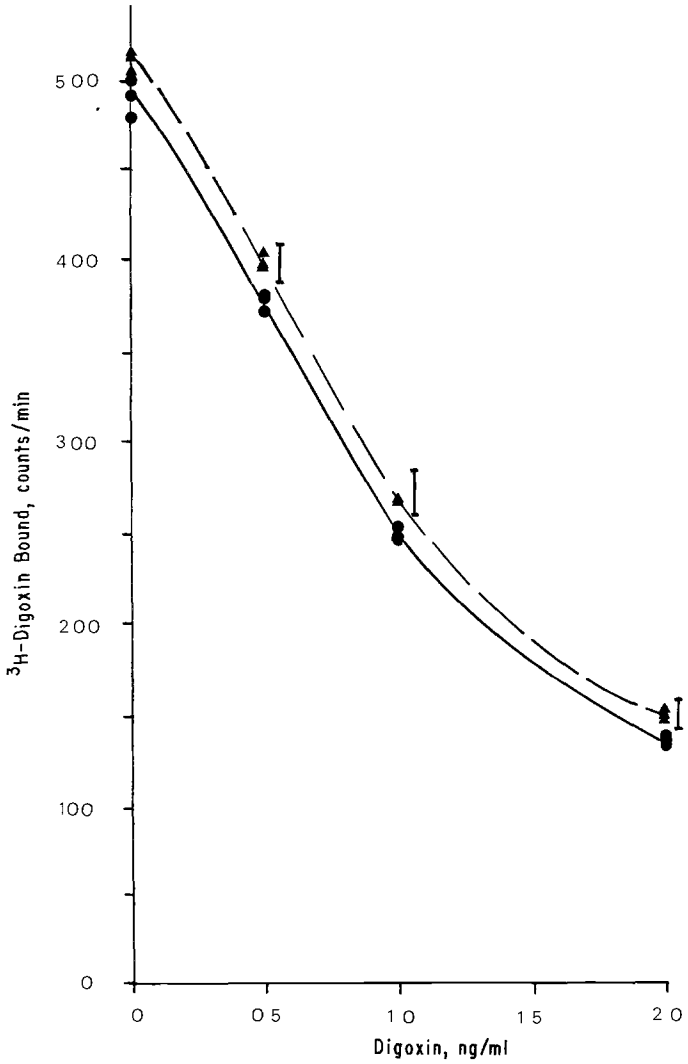

FIG. 2—*Response curves for standard digoxin dialyzed from normal horse serum* (closed circles with solid line) *and postmortem blood* (closed triangles with broken line). *±1 standard deviation limits are shown at 0.5, 1.0, and 2.0 ng/ml.*

percent intrabatch *and* interbatch, based on dialyzed digoxin standards in normal horse serum. This level of reproducibility, relatively high for radioimmunoassay, could be obtained with postmortem blood samples which were sufficiently mobile to allow accurate pipetting, and ultrasonication was instituted routinely to ensure this.

In quantitation methods involving extraction, it is often preferable to use a standard curve based on the extract rather than on the original medium, thus remaining independent of variation in the efficiency of extraction. A dialysis standard curve of horse serum digoxin standards is compared in Fig. 3 with a series of standards prepared in the antiserum itself (the two curves in the figure with closed symbols). It is important to note that these antiserum standards were allowed to equilibrate overnight before completion of the isotope dilution system with ^{3}H-digoxin. However, if antiserum standards were prepared in which antibody and unlabelled digoxin were not incubated together before the addition of ^{3}H-digoxin, there resulted a standard curve (open symbols in Fig. 3) of relatively low sensitivity at these reagent concentrations. (The antiserum dilution was such that zero standard count rates were normally 26-28 percent of total count rates.) The count rates of the standards prepared without preincubation were reduced by about 15 percent by the presence of 10 percent horse serum in the final aqueous solution.

It is likely that the high sensitivity achieved in the dialysis technique is largely due to the extensive preincubation of antiserum with unlabelled digoxin during the dialysis, followed by the relatively short incubation with ^{3}H-digoxin in which little of the antibody-digoxin association established overnight is reversed. Thus, the method may approximate more closely to back-titration of the free antibody present after the overnight incubation, rather than to a complete re-equilibration of antibody with the two competing digoxin species [*10*]. The advantages of the preincubation assay of digoxin in NHS have already been discussed in some detail [*10*].

In Fig. 3 the digoxin standard curve based on the antiserum (closed symbols) is of different slope to the response curve of the dialyzed standards in normal horse serum. This difference in slope is fairly reproducible, and if the two curves cross high enough (which depends on the dilution of the antiserum), the zero standard count rate of the antiserum standards is higher than the dialysis standards.

The reason for the difference in slope has not been established. It is not due to inhibition of the antiserum by some agent in the dialysis membrane, nor can it be attributed to change in concentration of the antiserum during dialysis (for example, as a result of osmotic effects, since antisera are made up to a standard weight after dialysis). However, the slope discrepancy does prejudice to some extent the normal preference for a standard curve based on the extract, particularly in view of the high reproducibility of the extraction.

Specificity

Table 1 shows the extent of cross-reaction of a number of compounds in dialysis assays performed on normal horse serum. The compounds fell into three groups: (1) those occurring in cases of suspected digoxin overdose submitted to operational forensic science laboratories, (2) those known to be bound by protein, and (3) steroids related to digoxin. Only the steroids reacted in the assay, and at concentrations well above those normally encountered in blood. The response curve of digoxin is indistinguishable from those of the cardiac glycosides digoxigenin and lanatoside C (Wellcome Reagents Ltd., personal communication).

In a case of suicide, 2.5 μg/ml of spirolactone or its metabolite aldadiene were found in postmortem blood by spectrofluorimetry, compared to the normal peak serum concentration of 0.4 μg/ml after therapy [*12*]. Yet even this spirolactone concentration

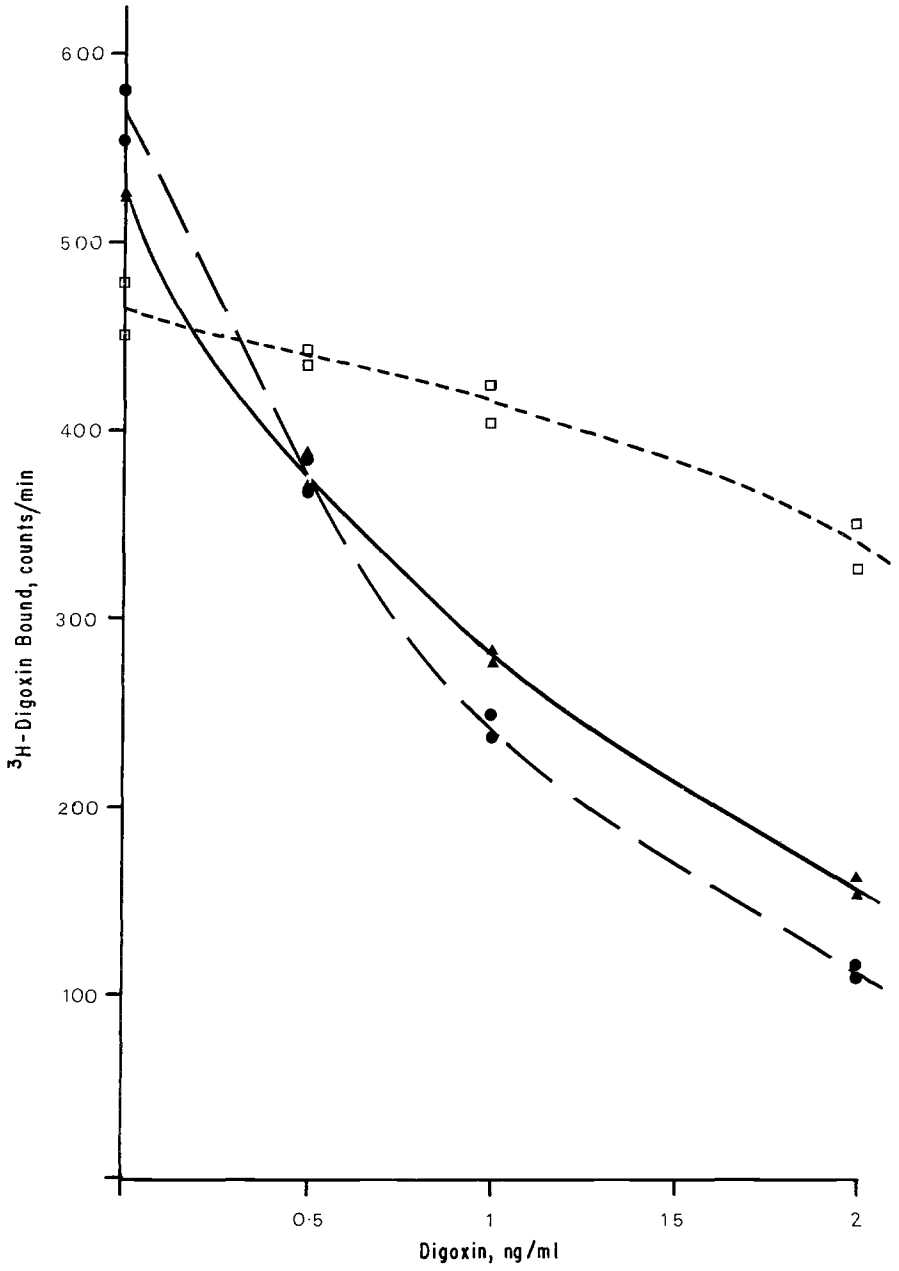

FIG. 3—*Response curves for standard digoxin:* (solid triangles with solid line) *digoxin dialyzed from normal horse serum;* (solid circles with broken line), *digoxin in antiserum, preincubated overnight;* (open squares with broken line), *digoxin in antiserum, not preincubated.*

TABLE 1—*Cross-reaction in dialysis radioimmunoassays.*

Compound	Concentration	Digoxin Equivalent, ng/ml in Dialysis Against Antiserum K3864	Antiserum K4996
Atropine	450 ng/ml	0	...
Hydrochlorothiazide	10 μg/ml	0	...
Oxytetracyline	200 μg/ml	0	...
Propranolol	2 μg/ml	0	...
Lasix	8 μg/ml	0	...
Trimethoprin	50 μg/ml	0	...
Sulphamethoxazole	250 μg/ml	0	...
Diazepam	1 μg/ml	0	...
Spironolactone	5 μg/ml	0.5	...
Prednisolone	5 μg/ml	0.13	...
Aldadiene	5 μg/ml	0.6	0.05
Prednisone	5 μg/ml	0.25	0.06
Digitoxin	40 ng/ml	0.80	0.45
Phenobarbitone	1 mg/ml	...	0
Aspirin	1 mg/ml	...	0
Ascorbic Acid	1 mg/ml	...	0
Amylobarbitone	1 mg/ml	...	0
Phenylbutazone	1 mg/ml	...	0
Paracetamol	1 mg/ml	...	0
Caffeine	1 mg/ml	...	0
Chlorpromazine	100 μg/ml	...	0
Testosterone	1 μg/ml	...	0.1
Progesterone	1 μg/ml	...	0.2
Hydrocortisone	1 μg/ml	...	0.1
Corticosterone	1 μg/ml	...	0.2

only reacted as 0.45 μg/ml of digoxin with the old antiserum (K3864). The extent of cross-reaction by 40 ng/ml digitoxin, a high therapeutic serum concentration, is not surprising in view of its structural similarity to digoxin. Response curves with the new antiserum (K4996) for digitoxin, prednisone, aldadiene, and digoxin, are shown in Fig. 4.

Attempts to Incorporate the Double Assay Principle

In a previous study, analogs of digoxin were found to differ in their rates of reaction with the antidigoxin serum, as well as in equilibrium position [*10*]. By utilizing differences in reverse reaction rate, a double assay was developed for serum digoxin, in which even digitoxin could be readily distinguished from digoxin by its performance in two assays carried out under different conditions. In the "simultaneous addition" assay, the unknown steroid is compared to a digoxin standard, with the ^{3}H-digoxin and competing steroid exposed to the antiserum simultaneously. In the second, preincubation assay, antiserum and unlabelled digoxin or test steroid were preincubated together before addition of the ^{3}H-digoxin. During a short (15-min) incubation the latter causes little re-equilibration in (unlabelled) digoxin-antiserum mixtures, yet largely replaces drugs other than digoxin from antibody associations established during preincubation. If the behavior of the unknown drug is not highly correlated to the digoxin standards in the two types of assay, it cannot be digoxin.

In these terms, the dialysis assay for postmortem blood is a preincubation. Antiserum Lot K3864 had been used in the double assay studies with serum, and the kinetics of ^{3}H-digoxin binding by the more specific antiserum Lot K4996 were examined as a

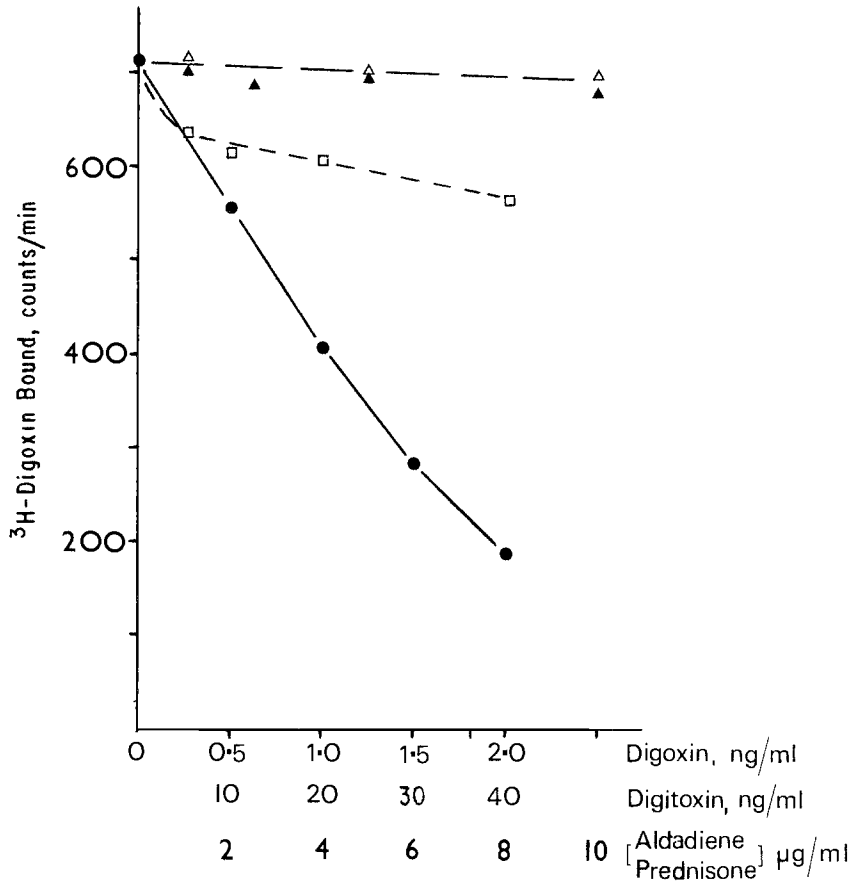

FIG. 4—*Response curves against antiserum K4996, standards dialyzed from normal horse serum:* (solid circles with solid line), *digoxin;* (open squares with broken line), *digitoxin;* (solid triangles with broken line), *prednisone;* (open triangles with broken line), *aldadiene.*

preliminary to the adaptation of the dialysis techique. The kinetic assays were carried out as described previously [*10*]. The rate of uptake of ^{3}H-digoxin (5 ng/ml) by antibody was compared in preincubation and simultaneous addition systems containing unlabelled digoxin (1 ng/ml) or digitoxin (80 ng/ml). The results in Fig. 5 show a similar differential dissociation of antibody-digoxin and antibody-digitoxin complexes to that found with the earlier antiserum.

In order to complement the established preincubation dialysis assay, several modifications of the reaction sequence were considered. In the first, as an approximation to the simultaneous addition type, the ^{3}H-digoxin was added to the antiserum before commencing dialysis, and reaction was terminated at leisure the next day, after equilibration to room temperature. A several-fold reduction in sensitivity (the BSI definition is used [*10*]) of both the digoxin and digitoxin response curves occurred. The failure to obtain a differential response was presumably due to the fact that the assay involved a *prior* addition of ^{3}H-digoxin to antiserum. In an alternative approach, the normal 15-min incubation of dialysis antiserum with ^{3}H-digoxin was extended by periods of up to 22 h, in order to approximate to a re-equilibration. As may be predicted from the data of Fig. 5, where the extent of binding of ^{3}H-digoxin in preincubation mixtures

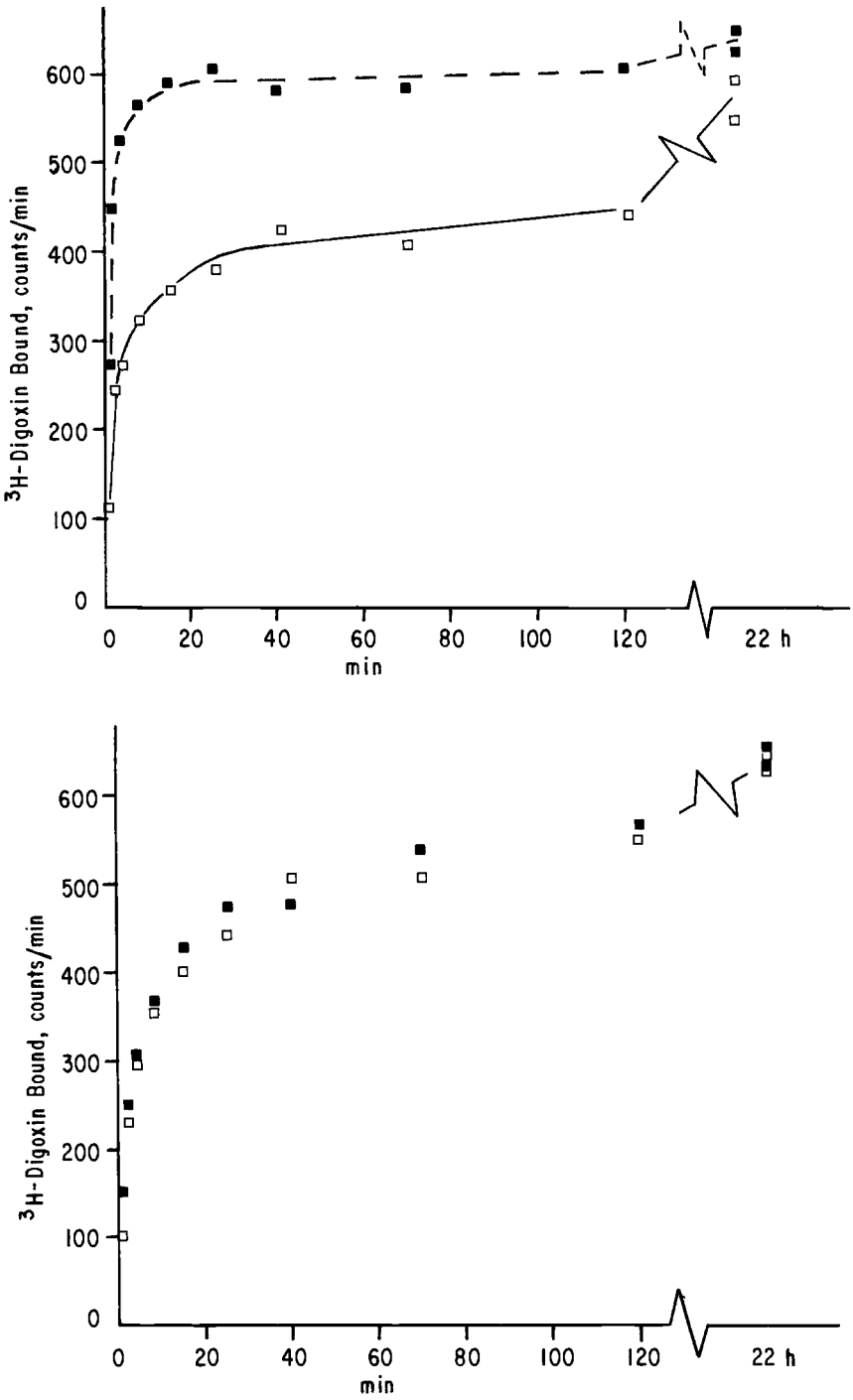

FIG. 5—*Kinetics of ^{3}H-digoxin binding by antiserum K4996 in "simultaneous addition"* (solid squares with broken line) *and preincubation* (open squares with solid line) *assays. Unlabelled steroid:* (top) *digoxin,* (bottom) *digitoxin.*

containing either unlabelled digoxin or digitoxin is seen to rise above the 15-min level after prolonged incubation, the response curves of standard digoxin and digitoxin diminished in sensitivity to the same extent.

However, as had been found with the serum assay method [*10*], a simultaneous addition assay provided a complementary system which enabled digitoxin to be discriminated from digoxin in postmortem blood. In the simultaneous addition assay, ^{3}H-digoxin from 100 ng/ml stock solution was added to the postmortem blood to 5 ng/ml, before dialysis overnight against antiserum. On the next day the antiserum was diluted sevenfold with buffer as usual, and 0.7-ml aliquots were removed to microcentrifuge tubes containing 0.1 ml of buffer. When convenient, charcoal suspension was added and the routine method completed. The results of simultaneous addition and "normal" dialysis assays of postmortem blood containing digoxin standards may be seen in Fig. 6. The assay results of blood samples containing 40 and 80 ng/ml of digitoxin are shown as intercepts on the two digoxin standard curves. The higher concentration of digitoxin, 80 ng/ml, equates with quite different digoxin concentrations under the two assay conditions. However, at 40 ng/ml, digitoxin could be more easily confused with digoxin, and here in particular discrimination is hindered by the fact that the reproducibility of the simultaneous addition method will be worse than the more sensitive preincubation dialysis technique [*10*]. Further studies may well show the simplest routine method consistent with high reproducibility to be to perform only the normal dialysis method, but to assay the unknown at two or more dilutions and thus exploit the difference in shape of the response curves of digoxin and of other drugs.

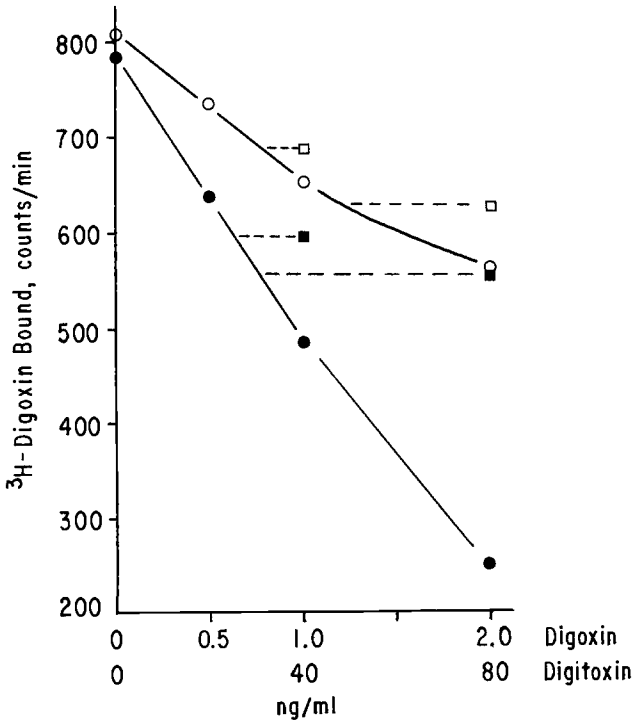

FIG. 6—*Normal preincubation dialysis assay of postmortem blood:* (solid circles with solid line), *digoxin;* (solid square), *digitoxin. Simultaneous addition dialysis assay:* (open circles with solid line), *digoxin;* (open square), *digitoxin.*

Response curves with the previous antiserum batch were made for a range of drugs [*10*], and although in simultaneous addition assays (of serum), differences from the digoxin standard curve were not great, higher concentrations of the drugs in the preincubation mode gave response curves quite different in shape from digoxin. In dialysis assays performed with the current antiserum, only digitoxin has been found to cross-react significantly, and here again multiple dilution analysis would certainly allow good discrimination between digitoxin and digoxin (Figs. 4 and 6).

Discussion

Digoxin is the cardiac glycoside in almost exclusive use in the United Kingdom, but in other countries digitoxin is used to a greater or lesser extent. Both radioactively labelled digitoxin and antisera to digitoxin conjugates are available commercially, and it seems likely that the forensic scientist could readily use the digitoxin radioimmunoassay as a dialysis technique. In fact, the dialysis principle, with the associated high sensitivity endowed by the preincubation aspect, may prove popular in radioimmunoassays of other drugs in blood and tissues taken postmortem. Furthermore, the double assay and multiple dilution principles should in many cases counteract the limitations of imperfectly specific antisera, and thus greatly reduce the delay between initiation of an immunization schedule and development of an acceptable monospecific assay.

Summary

A radioimmunoassay for digoxin in plasma has been modified for use with postmortem blood. Digoxin is extracted from the blood by dialysis against the antidigoxin serum. The only compound found to cross-react seriously in the assay is the closely related cardiac glycoside, digitoxin.

Acknowledgment

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