Chapter 18

Sample Preparation for Tritium Counting in the Application of the Digoxin Radioimmunoassay Technique to Lysed Blood

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INTRODUCTION

The cardiac glycosides, among which digoxin is almost exclusively prescribed in Great Britain, are extremely effective in the treatment of congestive heart failure and supraventricular tachyarrhythmias. Unfortunately there is a narrow margin between therapeutic and toxic doses, and since toxicity comprises aggravation of these cardiac conditions it was formerly impossible to distinguish between insufficient and over-dosage. To emphasize the seriousness of the situation, a recent survey revealed that toxicity results in 8 to 20% of hospital patients taking digoxin, with a subsequent mortality of 7 to 50% (Baller *et al.*).[1] The situation may improve as a result of the introduction of a radioimmunoassay (Smith *et al.*)[2] sufficiently sensitive to measure therapeutic digoxin levels in plasma, particularly now that the technique is available as a commercial kit.

This study on scintillation counting was a preliminary to the adaptation of the digoxin radioimmunoassay method to the analysis of whole blood taken at post-mortem examination. The high degree of haemolysis in post-mortem specimens poses a problem of colour quenching. The facility to examine such samples should be useful in forensic science, where overdose fatalities are sometimes encountered. Furthermore, by providing a retrospective measure to the clinician it may help to increase our understanding of the delicate balance between therapy and toxicity.

EXPERIMENTAL

Materials

The Triton X-100 used initially was purchased from Lennig Chemicals Ltd. Toluene, PPO and POPOP were the scintillation grades of Koch-Light Laboratories Ltd. Naphthalene, n-pentanol and Cellosolve were the laboratory reagent grades of B.D.H.; ethanol was their technical grade. Scintillation grade hyamine hydroxide and NE 250 solubilizer were purchased from Nuclear Enterprises Ltd., as were the scintillation cocktails NE 233, NE 216 and NE 250 (based respectively on toluene, xylene and dioxan).

Lanoxitest kits for the radioimmunoassay of digoxin were from Wellcome Reagents Ltd. 'Analar' hydrogen peroxide, 100 volumes 30% w/v, was purchased from B.D.H.

Table 1. Relative performance of 10 ml scintillator cocktails with 0.5 ml N.H.S. supernatant.

SCINTILLATOR COMPOSITION						c.p.m. as percentage of NE 250 standard	new pence/ 10^3 c.p.m.
Toluene/ POPOP/PPO[a]	NE 233	NE 216	NE 250	Naphthalene			
–	50%	–	–	–	50% Triton X-100	48.4[b]	2.0
60%	–	–	–	–	40% Cellosolve	35.7	3.1
52%	–	–	–	8%	40% Cellosolve	38.9	2.8
60%	–	–	–	–	40% Ethanol	36.2	2.5
52%	–	–	–	8%	40% Ethanol	51.2	2.2
80%	–	–	–	–	20% NE 520	42.6	14.0
72%	–	–	–	8%	20% NE 520	42.2	14.0
–	80%	–	–	–	20% NE 520	50.6	13.8
–	85%	–	–	–	5% Hyamine, 10% n-pentanol	57.7	4.3
85%	–	–	–	–	5% Hyamine, 10% n-pentanol	35.6	6.3
77%	–	–	–	8%	5% Hyamine, 10% n-pentanol	45.5	4.8
–	–	100%	–	–	– –	<1	–
–	–	–	100%	–	– –	121.0	3.0
5 ml of NE 250						100.0	1.8

[a] 0.4% PPO, 0.01% POPOP
[b] Counted with the sample changer temperature at 8.5°C.

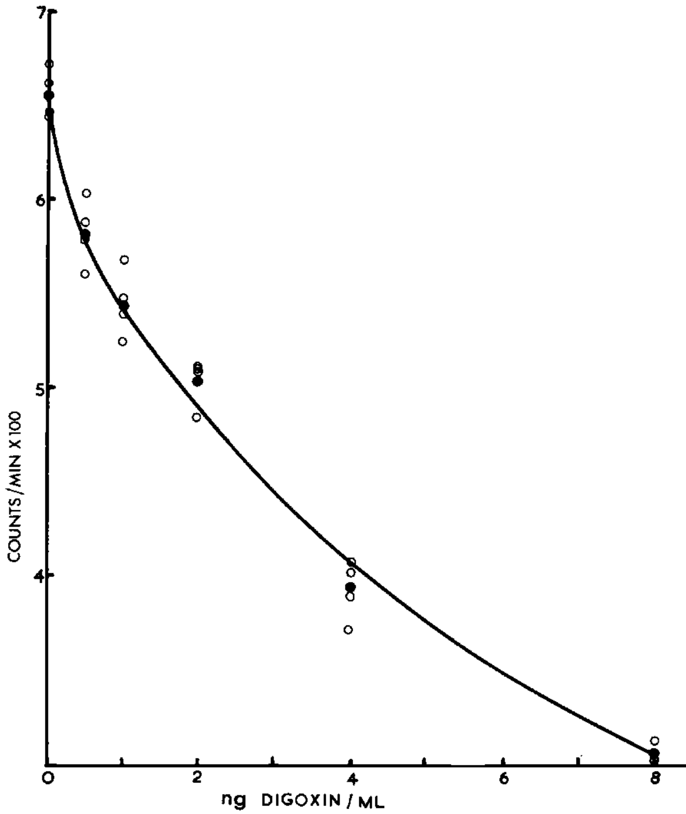

Fig. 1. Radioimmunoassay standard curve on lysed blood, using the Japax bleach technique.

Japax bleach is manufactured by Newlands Bros. and Mumford Ltd., 324 Harrow Road, London W9.

Counting methods

Scintillation counting of samples described in Table 1 and Fig. 1 was carried out in an Intertechnique SL 40 model, by kind permission of the Medical Research Council. The sample changer chamber in this instrument was at 12°C. The remaining data were derived in an Intertechnique SL 31, with the changer chamber at 8.5°C. 2×10^4 counts were collected routinely from each specimen.

Since the specific activity of the ^{3}H-digoxin was unknown, relative counting efficiencies are presented.

Radioimmunoassay method

The principle of the assay is that the reaction of a limiting quantity of antidigoxin serum with a quantity of ^{3}H-digoxin is inhibited by digoxin contributed by a test plasma, the degree of inhibition being related to the performance of a series of known digoxin solutions expressed as a standard curve. After the reaction has reached quasi-equilibrium, any ^{3}H-digoxin not bound to antibody is adsorbed onto activated charcoal, and the ^{3}H-

digoxin-antibody in the resulting supernatant counted in a liquid scintillation spectrometer.

Concentrated buffer, unlabelled and tritiated digoxin, anti-digoxin serum, and activated charcoal are provided in the Lanoxitest kit. The reaction mixture prescribed by the manufacturers consists of 0.1 ml plasma mixed with ^{3}H-digoxin and antiserum in a total of 0.8 ml. Normal horse serum (N.H.S.) is supplied for use in place of plasma in blank and standard tubes. After incubation, 0.2 ml of the suspension of charcoal are added, the mixture incubated further and then clarified by centrifugation in a bench centrifuge. 0.5 ml portions of the final supernatant were routinely counted for tritium in this laboratory, assays being performed at least in triplicate. When several scintillation systems were under comparison, a single large reaction mixture was utilized, and 0.5 ml portions of the supernatant dispensed to individual counting vials. These reaction mixtures contained either N.H.S., outdated blood bank whole blood lysed by a single freezing/thawing cycle, or post-mortem blood believed to be digoxin-free.

Choice of a scintillator for N.H.S. supernatants

A variety of scintillator cocktails were examined for use with N.H.S. supernatants. The latter displayed only a faint straw colouration, and thus colour quenching was not expected to be a serious problem. Samples were loaded into the scintillation counter immediately after brief shaking, with the exception of Triton emulsions which were first allowed to stand in a 55°C oven for 5 min. The relative performance of the systems may be seen in Table 1. Count rate has been expressed as a percentage of that obtained with 5 ml of NE 250, the system chosen as a reasonable compromise of efficiency, economy and ease of use.

Samples prepared in NE 250 were allowed to equilibrate for several hours before counting. Even so, count rates continued to rise, there being a further increase of about 5% by 24 h, and thus where great accuracy was required samples were not counted until 24 h after preparation.

Scintillation counting of haemolysed supernatants

Replacement of N.H.S. by lysed whole blood in the radioimmunoassay resulted in serious quenching, presumably due to the pronounced redness of the supernatant and subsequent scintillation mixture. In 5 ml NE 250 the count rate was reduced to 30% of that obtained with samples prepared in parallel with N.H.S., and in 10 ml NE 250 a relative count of 54% was achieved.

Three inexpensive decolourants were considered, hydrogen peroxide, chlorine water and a commercial hypochlorite bleach, 'Japax'. Since the standard 5 ml of NE 250 was rather intolerant of a significant increase in aqueous content over the normal 0.5 ml (an additional 0.5 ml of distilled water reducing the count rate by 20%), the minimal necessary volumes of the decolourants were investigated. Reasonable volumes of chlorine water had little effect even after prolonged incubation. The maximal bleaching effect was obtained with 0.20 ml of hydrogen peroxide or 0.225 ml of Japax within a few minutes at room temperature, the resulting fluid being a rather deeper straw colour than N.H.S. supernatants. As a precaution the pH of samples was checked, and it was necessary to add 0.025 ml of 3 M acetic acid to adjust the Japax-treated solutions to about pH 6.5. Supernatants decolourized with hydrogen peroxide displayed marked inter-sample variation, with replicas differing by up to 70%, and this method was considered no further. The supernatants bleached with Japax counted reproducibly at 77% of the efficiency of com-

parable N.H.S. supernatants; in 10 ml NE 250 they counted at 95%. Normal background count rates were registered in the absence of ^{3}H-digoxin.

Once bleached haemolysed samples had been allowed to equilibrate with the temperature of the scintillation spectrometer, count rates were stable for at least 72 h. The rapid equilibration contrasts with the 24 h period necessary for N.H.S. supernatants counted directly in NE 250 and is probably a consequence of the increase in homogeneity conferred by the bleaching process. In fact, Japax-treated samples in NE 250 formed emulsions which separated into two clear phases on standing, the aqueous phase retaining the yellow colour of the bleached haemolysed supernatant. In contrast, untreated N.H.S. supernatants in NE 250 resulted in a flocculent precipitate under a single clear liquid phase. Treatment of N.H.S. supernatants with Japax bleach before counting in NE 250 resulted in a bilayer system with the rapid equilibration found for haemolysed supernatants.

As a final test, a standard curve was prepared from radioimmunoassays based on lysed whole blood, using the Japax treatment. The results are shown in Fig. 1. The criteria for a successful assay were in general satisfied. Replicas agreed well, with coefficients of variation between the various sets of quadruplicates varying from 1.9 to 4.7%.

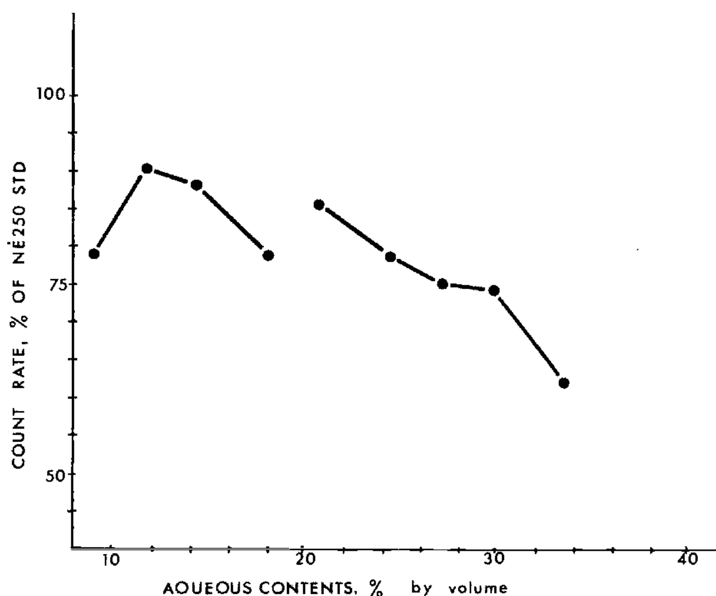

Fig. 2. Counting performance of N.H.S. supernatants in Triton emulsions with varying water content.

Further examination of Triton emulsion systems

Because of the advantages accredited to Triton emulsion counting of tritium in the recent literature (Williams and Florkowski,[3] van der Laarse,[4] Williams,[5] Turner[6]) this type of system was reconsidered. First it was necessary to ascertain to what extent the relatively poor efficiency encountered in the 1 : 1 Triton X-100 : NE 233 mix (Table 1) had been due to the technical quality of the Triton used. Substitution of scintillation grade Triton (Intertechnique Ltd.) in this system, in fact, only increased the efficiency

Table 2. The counting of post-mortem supernatants.

Bleach (ml)	3 M Acetic acid (ml)	Additional water (ml)	NE 250 (ml)	Triton/toluene (ml)	c.p.m. (mean of Triplicates)
–	–	–	5.0	–	420
–	–	–	10.0	–	707
–	–	0.5	–	4.5	61
0.225	0.025	0.25	–	4.5	580
0.225	0.025	–	5.0	–	743

to 56% of that in 5 ml NE 250. Increasing the water content in stages up to 23% by volume only served to reduce the count rate, finally to 49% of the NE 250 figure.

However, such a high Triton content has not been found to be ideal for efficient counting[5] and so a cocktail was prepared comprising 2 : 1 analar toluene : scintillation grade Triton with 0.7% PPO and 0.035% POPOP. The aqueous contribution of 0.5 ml N.H.S. supernatant was augmented to varying degrees by the addition of distilled water in a total vial content of 5.5 ml. As seen in Fig. 2, the resulting discontinuous curve is very similar to those reported previously.[3,5] The maximum count rate encountered was 90% of the NE 250 system at 12% aqueous content.

Of the mixtures shown in Fig. 2, that with an aqueous content of 18.2% was selected for a detailed stability study. Repeated counting of such samples showed that the count rate remained within the ±2 SD limits for 48 h after initial temperature equilibration. This system was applied to Japax-bleached supernatants based on actual post-mortem blood. As may be seen in Table 2, bleached samples counted well in the emulsion, the efficiency relative to the bleached NE 250 system (77%) being almost identical to the relative performance of untreated N.H.S. supernatants in Triton and NE 250 (Fig. 2). There was no evidence of chemiluminescence. The Triton system was particularly susceptible to colour quenching by untreated post-mortem supernatants.

It is noteworthy that our calculated cost of 1.1 new pence/10^3 counts for N.H.S. supernatants counted in 4.5 ml of this Triton/toluene cocktail is better than any of the costs listed in Table 1.

REFERENCES

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DISCUSSION

H. Dobbs: Comment: At Reckitt and Colman we put Harpic in our lavatories, but I have never thought of putting it in our counting bottles!