

Application of Glass Ampoules in the Assay of
 β -Radioactivity in Small Biological Samples

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ABSTRACT

Some applications of sealed glass ampoules, in the 1-5 ml range, to the radioassay of ^3H , ^{14}C and ^{35}S in biological samples were examined. After sample preparation, the ampoules were placed in cylindrical Perspex holders, machined to hold the ampoules in a central position, and radioassayed. The results were compared with those obtained with similar samples prepared in conventional 20 ml glass screw-cap vials.

Counting efficiencies and E^2/B values, especially with ^3H , were higher in ampoules than in vials and samples could be measured with similar precision in the two systems. For monitoring quenching, the automatic external standard system could be applied satisfactorily with ampoules even though the ratios were lower than for vials (with unquenched samples: 0.85 for 20 ml vial; 0.42 for 1 ml ampoule).

For the assay of tritiated water (0.2 ml), a system using 2 ml ampoules and a toluene-scintillator - Triton X-100 mixture (balance point counting efficiency 25.6%) was superior to one using 20 ml vials and a toluene-scintillator-ethanol solution (efficiency, 13.3%).

Satisfactory procedures for the assay of β -radioactivity in sheep blood plasma, urine and faeces, and in competitive protein binding assays of steroid hormones have also been developed.

INTRODUCTION

The assay of β -radioactivity in small biological samples is usually carried out with screw-cap glass or polyethylene vials ranging in size from 20 ml to the more recently available 5 ml "mini vial". Small plastic bags have also been used successfully by Gupta (1). In our laboratory, sealed glass ampoules of 1 to 5 ml capacity have been used to assay samples containing [^{14}C]formic acid which, because of its volatility, slowly escapes from screw-cap vials (2). Small ampoules have other advantages, especially in reducing sample costs and problems associated with the storage and disposal of samples (3, 4).

Since samples in the 1-5 ml range are large enough for many other applications of liquid scintillation spectrometry, we have examined the feasibility of using ampoules for assaying ^3H , ^{14}C and ^{35}S in various types of biological samples commonly studied in this laboratory.

MATERIALS AND METHODS

Scintillation solutions.- Toluene containing p-terphenyl (3g/l) and dimethylPOPOP (0.1g/l) was the basic solution used in most experiments (Solution A). For aqueous samples Solution A was mixed with the emulsifying agent Triton X-100 (5). Solution B comprised two parts of Solution A to one part of Triton X-100 and Solution C seven parts of Solution A to six parts of Triton X-100. Solutions D and E contained 2, 5-diphenyloxazole (PPO) (4g/l) and dimethylPOPOP (0.1g/l) in toluene plus ethanol (D, 45% v/v, E, 40% v/v).

Two reference liquid scintillation solutions were prepared by adding n-[1- ^{14}C]hexadecane (1.1 ml; 0.88 μCi) and n-[1,2- ^3H]hexadecane (1.3 ml; 2.18 μCi) respectively to Solution A (500 ml). These solutions were dispensed with an automatic Hamilton Precision Liquid Dispenser fitted with a 5 ml gas-tight adjustable syringe. The coefficient of variation due to the dispensing errors ranged from 0.02% (5 ml) to 0.09% (1 ml).

Sample preparation.- Glass ampoules (1, 2, 3 and 5 ml) and vials (20 ml screw-cap) were obtained from Australian Glass Manufacturers Ltd. (Sydney, Australia). After adding the sample and scintillation solution each ampoule was transferred to a revolving brass holder and sealed using a gas-oxygen hand torch. It should be

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emphasized that we perform this step in a well ventilated hood away from stock scintillation solutions and other flammable materials.

In preliminary tests the ampoules were placed in vials for counting and the effects of water coupling (6) and of positioning the ampoules in the vials were studied. In subsequent experiments each ampoule was placed in a cylindrical Perspex (polymethylmethacrylate) holder (5 cm long, 2.75 cm diameter) in a central well whose depth was such that the ampoules' mid-points were on the same horizontal plane. No water coupling was used with these holders.

In all experiments (except the preliminary one and the progesterone assay) groups of 10 samples were prepared for each comparison of ampoules and vials. For studies of samples in aqueous solutions tritiated water (0.27 μ Ci/ml) and aqueous solutions of L-[³H]leucine (1mCi/ml), L-[¹⁴C]lysine (2 μ Ci/ml) and L-[³⁵S]cystine (0.5 μ Ci/ml) were used. ¹⁴C-labelled plasma (7nCi/ml) was separated from blood collected four days after administering L-[¹⁴C]cystine (1mCi) intravenously to a sheep. Plasma containing L-[³H]leucine (1.6 μ Ci/ml) was used to study the radioassay of tritium in plasma.

To test the applicability of ampoules for the assay of radioactivity in sheep urine, two specimens were selected, one lightly coloured and the other much darker, to represent extremes of colour. To 50 ml of each, L-[¹⁴C]lysine or L-[³H]leucine was added giving solutions with specific radioactivities of about 0.05 μ Ci/ml and 0.15 μ Ci/ml respectively.

To measure ³⁵S in faecal samples, L-[³⁵S]cystine (0.2 ml; 0.1 μ Ci) was added to ampoules (5 ml) containing homogenised sheep faeces (50 mg). The samples were dried at 105°C (2 hr), and heated with 1 ml of an oxidizing solution (3 volumes conc. HNO₃ plus 1 volume 60% HClO₄ with 10g Mg(NO₃)₂ per 100 ml mixture) (7) in an aluminium block on an electric hot plate. The temperature of the block was raised to 260-280°C (during 1 hr) and the samples digested further until only a white crust remained. Then they were cooled, water (1.5 ml) added to dissolve the solid, Solution C (2 ml) added and the ampoules were sealed. To determine whether any ³⁵S was lost during oxidation, similar samples were prepared except that the L-[³⁵S]cystine (in 1.5 ml H₂O) was added after the oxidation step.

To test the application of ampoules for measuring steroids in plasma an assay method for progesterone in sheep plasma (8, 9) was chosen. Dog plasma containing [1,2-³H]corticosterone was the competitive protein binding agent. The assay procedure involved the setting up of a binding curve covering the range 0 to 8 ng of progesterone. The labelled reagent was added to a tube containing the unlabelled progesterone. After incubation at 40°C for 15 minutes the proportion of label in the protein-bound fraction (1.5 ml) was collected from a Sephadex G25 fine column (30 x 8 mm diam.) in either a 20 ml vial or 5 ml ampoule containing 0.1 ml 5N HCl. Solution A (7.5 and 3.5 ml respectively) was added and after thoroughly shaking the samples and allowing them to settle, their counting rates were determined.

Radioassays.- A Model 3375 Packard Tri-Carb Liquid Scintillation Spectrometer, refrigerated at 30°C, was used. The optimum gain setting for balance-point operation (RED channel discriminators 50-1000) was selected for each series of samples using the technique of Neame and Homewood (10). The GREEN channel was operated in the integral mode (100% gain, 20-∞). In each series quenching was determined using the automatic external standard (AES) ratio.

The counting efficiency (%) was determined for the plasma, urine and faeces samples by several methods. For plasma a dried sample was combusted in an oxygen flask system (11). The counting efficiency of the solutions containing the combustion products was calculated after adding radioactive hexadecane as an internal standard ('spiking'). The plasma and urine counting efficiencies were also measured by spiking samples with [¹⁴C] or [³H]hexadecane or with tritiated water.

Some faeces samples with no added ³⁵S were oxidized (7) and spiked with [¹⁴C]hexadecane. The error in using a ¹⁴C spike instead of ³⁵S is small (12). From the curve relating AES ratio and counting efficiency the counting rates of the oxidized faecal samples containing ³⁵S were corrected for quenching.

RESULTS

Unquenched ¹⁴C and ³H Samples.- In a preliminary experiment 5 ml ampoules containing 5, 4, 3, 2 and 1 ml of Solution A and containing [¹⁴C] or [³H]hexadecane, 10μg/ml, were tested with the ampoules placed in 20 ml vials. Other

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ampoules (3, 2, 1 ml capacity containing 3, 2 and 1 ml respectively) were similarly prepared. The ampoules were compared with vials containing 20 ml of the radioactive solution. Since there were only small differences in balance point gain setting (maximum differences, 0.5% for ^{14}C and 5% for ^3H) a mean value (9% for ^{14}C , 55% for ^3H) was selected for all ampoules in this experiment. Vials were counted with 10% (^{14}C) or 55% (^3H) gain.

The counting efficiency was higher with the ampoules than the vials. For ^{14}C the improvement was about 2% and for ^3H about 10%. Water coupling increased the integral counting efficiency for ^{14}C by 0.6% and for ^3H by 2-3%. There was no change in counting efficiency with different volumes in 5 ml ampoules. The AES ratio decreased with a decrease in ampoule size. The variability in measuring the AES ratio was greater when the ampoules were not held in a central position.

The reproducibility of the results was studied more carefully with ampoules accurately positioned in Perspex holders. A series of each ampoule size and of the vials were prepared with the corresponding volumes of the standard solutions. Balance point gain settings were 9% for ^{14}C and 55% for ^3H . A minimum of 2×10^5 counts was recorded for each measurement of counting rate.

The balance point and integral counting efficiencies for ^{14}C in ampoules were about 2% greater than the corresponding values for vials (Table I). There was also an increase (about 0.5%) in efficiency with decreasing ampoule volume. The AES ratio, while decreasing from 0.849 for 20 ml vials to 0.421 for 1 ml ampoules was measured accurately enough (coefficients of variation 1.1 - 1.8%) in the smaller ampoules to enable quenching to be monitored.

The ^3H counting efficiencies showed larger changes (Table I). The efficiencies were higher by 6% (5 ml) to 11% (1 ml) for the ampoules compared with vials.

The background counting rates (Table II) at the balance points were reduced from about 28 to 20 cpm by using ampoules instead of vials. However, the main contribution to the background counting rate was attributable to the ampoule holder and counting system, and not to the scintillation solution itself. When maximum counting rates are required integral counting can be used. With a 20 ml vial the background cpm was trebled and with a 1 ml ampoule doubled when compared with the balance point background values shown in Table II.

The effects of the increased counting efficiency for the low-volume ampoules (Table I) and the reduced background counting rates are reflected in the high E^2/B values, up to 377 for ^{14}C and 165 for ^3H , under balance point conditions (Table II). These values for both ^{14}C and ^3H in 1 ml ampoules were about 30% higher than E^2/B values for the sealed instrument standards supplied by the manufacturer.

Measurement of Tritiated Water.- The use of tritiated water to measure total body water is now a routine procedure (13). Two 'cocktails' (Solutions E and B) with 5 ml and 2 ml ampoules respectively were compared with the cocktail (Solution D) previously used in this laboratory.

The results (Table III) showed that the counting efficiency for tritium in the 2 ml ampoules was approximately twice the efficiency observed with the 20 ml vials and about 1.6 times the value observed with the 5 ml ampoules. All systems showed stable counting rates over a period of three weeks.

Radioassay of ^{14}C and ^3H in Sheep Plasma and Urine.- The system routinely used in this laboratory for assaying β -radioactivity in plasma or urine has been based on a 1 ml sample which is mixed with water (6.5 ml) in a vial to which Solution C (10 ml) is added. Besides forming a stable sample (gel) the dilution of the urine with water considerably reduces its colour quenching. Colour quenching is usually not a problem with plasma. However, a gel system is necessary otherwise the plasma proteins slowly precipitate from the cocktail. Since it is difficult to scale down these systems to the ampoule volume (5 ml) other combinations of the sample components were tested.

On the basis of these tests the following cocktails were adopted: for plasma, 1 ml sample, 1 ml water and 3 ml Solution C; and for urine, 0.5 ml sample plus 4.5 ml Solution B. A small amount of material slowly precipitated from the urine cocktail but this had little effect on the counting efficiency. The results (Table IV) show that higher counting efficiencies and a smaller effect of quenching were obtained for urine when the cocktail adopted for 5 ml ampoules was used instead of the one used for the 20 ml vials. The counting efficiencies with plasma were about the same in the two systems.

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Radioassay of ^{35}S in Sheep Faeces.- The results from the digestion of 10 faeces samples containing ^{35}S showed that the counting efficiencies (based on [^{14}C] hexadecane) ranged from 62.5 to 66.8%. This comparatively wide range of values was due to variable quenching. When corrected for quenching the mean efficiency corresponding to the highest AES value (0.200) was $65.8(\pm 0.4 \text{ S.D.})\%$. On comparing samples in which the same amount of ^{35}S was added before and after performing the oxidation it was found that the mean recovery of added radioactivity was $101.9(\pm 0.9 \text{ S.D.})\%$.

Competitive Protein Binding Assay for Progesterone.- Separate binding curves were prepared for the vials and ampoules. The mean cpm of triplicate samples was graphed against 0, 0.25, 0.5, 1, 2, 4, 8 ng of progesterone. The counting rates obtained with the vials and ampoules were similar (ampoules $95.4(\pm 3.3 \text{ S.D.})\%$ of vials) and gave substantially the same results ($r = 0.9998$) for equal masses of added progesterone. When the HCl was omitted, there was a larger difference between the counting rates obtained with the two systems, which differed only in the amount of scintillator added. The radioactivity measured in a sample of the aqueous phase indicated that a larger proportion (about 20%) of tritium remained in the samples which had not been treated with acid.

DISCUSSION

The above results show that a wide variety of biological samples containing β -radioactivity can be assayed as satisfactorily in sealed glass ampoules as in conventional screw-cap vials. Although the size of the sample is obviously restricted by the size of the ampoule, in our experience the procedures described for the assay of radioactive plasma, urine and faeces samples are sensitive enough for most tracer experiments, even with animals as large as sheep. Any loss in sensitivity due to using smaller samples is counteracted, at least to some extent, by the higher counting efficiencies, lower background counting rates, and smaller effects of quenching in ampoules.

The AES ratio was lower for the smaller samples. In the spectrometer used the ratio of the upper portion of the β -spectrum due to Compton electrons produced by the γ -source is compared with the whole spectrum. With the

smaller sample volumes a greater proportion of the more energetic γ -rays pass through the scintillation solution without producing electrons. This presumably explains the reduction in the AES ratio. The precision in measuring the AES ratio for 20 ml vials has been studied by Stanley (14) who showed that the major error is caused by irregular vial geometry and vial positioning within the detector. When the same vial was assayed repeatedly with the sample changer operating in the normal cycling mode Stanley found that the coefficient of variation in the measurement of the AES ratio was 0.7%. This may be compared with the value of 1.1% which we observed with a set of ten 20 ml vials.

For many experiments the solubilization or oxidation of the sample can be conveniently performed in the ampoule before adding the scintillation solution. Losses of radioactive material during digestion are less likely with ampoules than with vials. The use of sealed ampoules eliminates losses of solvent or sample on storage or on mixing samples such as those containing Triton X-100 which have to be shaken vigorously to form a gel. With some batches of screw-cap vials in which poorly fitting caps were supplied, such losses have caused considerable inconvenience in this laboratory. The use of ampoules also eliminates another potential source of error - cap luminescence - which is sometimes possible when screw-cap vials are used (15). Another advantage in using ampoules is that toluene vapour does not accumulate in the sample changer of the scintillation counter. Ampoules are easier to store and considerably reduce the problems in disposing of large volumes of scintillation solution.

In analytical applications of liquid scintillation spectrometry, such as radioimmunoassays and allied techniques, much larger numbers of samples are envisaged in future research. For such testing, ampoules would be useful because they can be filled and sealed by fully automatic and readily available machines. With the manual technique of sealing ampoules which we have used, experienced operators can easily prepare and seal more than 100 per hour. We have sealed many thousands of ampoules by the method described, without experiencing any problems, but again we emphasize that appropriate precautions should be taken to avoid a fire.

A difficulty arises when it is necessary to spike samples in sealed ampoules after their counting rates have

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determined. It is possible to open and re-seal ampoules after adding a spike, but this is not as easy as removing and replacing a screw-cap. An alternative is to prepare duplicates of samples covering the range of quenching being studied and to add the spike before the initial sealing.

Improvements could be made in the counting efficiencies which we described, by using better scintillators and solubilizers. However, our aim has been to reduce costs as much as possible in a laboratory where large numbers of samples (currently about 100,000 per year) are assayed. Ampoules containing 5 ml scintillation solution cost about a fifth as much as the cheapest available screw-cap vial containing 20 ml solution.

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TABLE I. Liquid scintillation counting of [^{14}C] and [^3H]hexadecane in vials and ampoules.

Volume	AES Ratio	Counting Efficiency (%)			
		Balance Point		Integral	
		^{14}C	^3H	^{14}C	^3H
20 ml*	0.849(1.1)	84.0	42.5	91.8	43.7
5 ml†	0.762(1.1)	85.5	48.4	93.6	49.9
3 ml	0.666(1.2)	85.6	49.9	93.6	51.5
2 ml	0.581(1.1)	85.9	50.3	93.8	51.9
1 ml	0.421(1.8)	86.0	53.2	94.1	55.1

The sample containers were either 20 ml vials (*) or 5, 3, 2 or 1 ml ampoules (†).

The balance point settings were for ^{14}C , 9%, 50-1000 and ^3H , 55%, 50-1000.

The results are means for 10 samples with 2×10^5 counts recorded for each. The coefficients of variation of the counting efficiency ranged from 0.2 to 0.4 (^{14}C) and 0.8 to 1.8 (^3H). The values in brackets are the coefficients of variation for the AES ratio.

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TABLE II. Background counting rates and figures of merit (E^2/B) for unquenched ^{14}C and ^3H samples at balance point.

	Carbon - 14		Tritium	
	Background cpm	E^2/B	Background cpm	E^2/B
20 ml*	28(17)	257	29(26)	63
5 ml†	20(20)	360	24(21)	100
3 ml	20(20)	362	20(19)	126
2 ml	20(20)	372	19(18)	138
1 ml	20(19)	377	17(16)	165
Standard‡	24	303	20	120

The sample containers were 20 ml vials (*), 5, 3, 2, 1 ml ampoules (†) and the instrument sealed standard (‡).

The balance point settings were for ^{14}C , 9%, 50-1000 and ^3H , 55%, 50-1000.

The total number of counts was 10^3 (for each of 10 samples). Values in brackets were the counting rates when vials or holders plus ampoules without added scintillator were used. The counting rates without holders were 16 and 6 cpm respectively for ^{14}C and ^3H .

TABLE III. Radioassay of tritiated water.

Volume	Solution	AES Ratio	Counting Efficiency (%)	
			Balance point	Integral
20 ml*	D	0.441(1.3)	13.2(2.2)	14.2(2.1)
5 ml†	E	0.399(3.3)	16.1(4.4)	17.1(4.2)
2 ml†	B	0.372(3.6)	25.6(3.4)	26.7(3.2)

The sample containers were 20 ml vials (*), with 10 ml of Solution D and 5 and 2 ml ampoules (†) with 5 ml of Solution E and 2 ml of Solution B respectively.

The balance point setting was 80%, 50-1000.

The results are means for 10 samples with 2×10^4 counts recorded for each. Coefficients of variation are shown in brackets.

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TABLE IV. Radioassay of plasma and urine samples.

Sample	Volume	Carbon - 14		Tritium	
		E [‡]	AES Ratio	E	AES Ratio
Plasma	20 ml*	67.3	0.322(2.8)	5.8	0.271(1.1)
	5 ml [†]	67.5	0.252(1.1)	5.7	0.198(5.7)
Urine-1	20 ml	56.6	0.228(1.9)	3.7	0.211(4.2)
	5 ml	70.3	0.358(3.4)	13.2	0.371(3.0)
Urine-2	20 ml	40.7	0.086(3.6)	1.6	0.089(4.0)
	5 ml	64.2	0.242(4.9)	9.2	0.246(4.8)

The sample containers were 20 ml vials (*) with 1 ml of plasma or urine, 6.5 ml water and 10 ml Solution C, 5 ml ampoules (†) with 1 ml plasma, 1 ml water and 3 ml Solution C, or 0.5 ml urine and 4.5 ml Solution B.

Urine-1, -2 were the light and dark coloured samples respectively.

‡Counting efficiency (E) was determined at balance point settings for ¹⁴C of 40%, 50-1000 and ³H, 100%, 50-1000.

The results are means for 10 samples with 2×10^5 counts recorded for each. Coefficients of variation are shown in brackets.

