

## Chapter 9

# Sample Preparation Techniques for Tritium Counting in Biological Systems

P. Johnson, T. J. Rising and B. R. Twite

*Drug Development, Hoechst Pharmaceutical Research Laboratories, Milton Keynes  
Buckinghamshire, England*

### INTRODUCTION

It was not until the late 1950's that the first commercially available automatic liquid scintillation counters appeared on the market, and the following decade saw considerable improvements in instrument design and in data presentation.<sup>1</sup> In the last few years, instruments designed for routine sample preparation have been introduced, particularly sample oxidisers.

A number of different types of sample oxidisers are currently available which provide samples for liquid scintillation counting from labelled biological material and which also separate  $^3\text{H}$  and  $^{14}\text{C}$ . The principle of operation is the same in all models, namely that the sample is combusted totally to water vapour and carbon dioxide. The water vapour is condensed and collected in a counting vial, and the carbon dioxide is absorbed in a suitable trapping agent. The vials are then automatically filled with scintillator.<sup>2</sup> Thus, biological samples containing either  $^{14}\text{C}$  or  $^3\text{H}$  or both can be processed in this way.

In some cases, it is insufficient simply to ascertain the total amount of radioactivity in a sample, as, for instance, following the administration of tritium-labelled compounds to animals and man. Biological exchange processes can lead to the incorporation of tritium into body water. This results in the half-life of the compound in the body being overestimated, misleading tissue retention data and incorrect kinetics. We have overcome this problem by using a Packard Tri-Carb 306 Oxidiser which has been modified to allow differentiation between tritium present as body water and tritium present as parent compound or metabolites.

### THE MEASUREMENT OF TRITIATED WATER USING A MODIFIED SAMPLE OXIDISER

The oxidiser has been modified by the addition of an ignition switch which cuts the electrical supply to the heating coil, thereby preventing ignition of the sample in the combustion chamber (Fig. 1). Samples are held in a paper combustion cone. Tissue samples are cut into small pieces and pulped against the sides of the combustion cone using a spatula and liquid samples are absorbed onto fluted filter

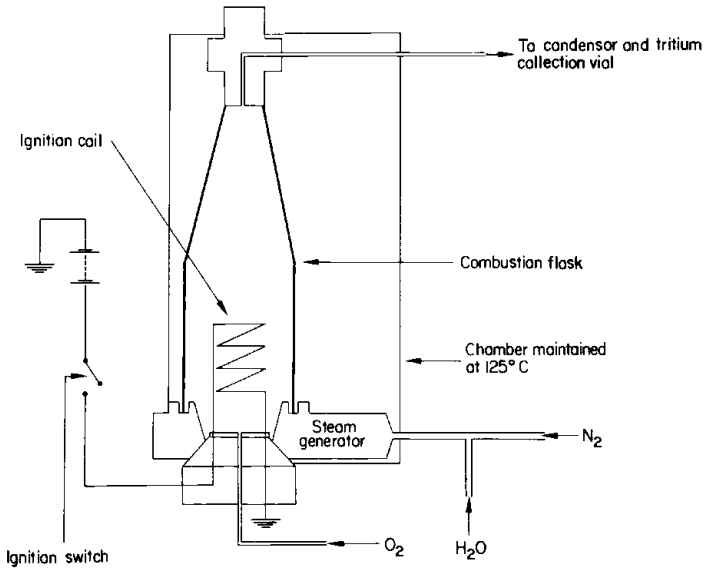


Fig. 1. Diagram of the combustion chamber of a Packard Model 306 Sample Oxidiser.

paper. When the programme is started, the sample is lifted into the combustion chamber, which is maintained at 125 °C. Provided a hole is made in the base of the combustion cone to allow the hot gases to circulate, a 200 mg sample will dry in 6 min, and the resultant water vapour is collected in the tritium collection vial. The ignition is then re-set and about 20  $\mu$ l of water is added to the now dry sample to slow down the burning process. When the programme is restarted, the sample combusts in the normal way. The drying step gives a measure of tritiated water in the sample and the combustion step gives a measure of the tritium content of the compound and any metabolites.

## VALIDATION OF METHOD

We have checked the efficiency of drying in two ways, using a wide range of biological samples. First, we have compared the weight loss after drying using the oxidiser with the weight loss after freeze-drying using a conventional Virtis freeze-dryer (Table 1). The weight loss figures, drying either with the sample oxidiser or with the freeze-dryer, give percentage weight losses that differ by no more than 3%. Second, we have measured residual radioactivity by combustion of samples previously spiked with tritiated water and dried with the oxidiser (Table 2). No more than 4% of the original radioactivity is found in the samples after the drying step. Therefore, the modified sample oxidiser allows very efficient drying of biological samples.

However, although the trapping efficiency is 98% when a sample containing tritium is combusted with the oxidiser, only 85% of the radioactivity is retained

in the tritium collection vial when a sample is dried (Table 3). Three percent of the radioactivity remains in the sample and appears in the tritium collection vial on combustion of the sample. If two acetone/dry ice traps are put in series on the gas line, a small amount of radioactivity is retained, whilst 7-8% is collected in the effluent from the wash cycle, giving an overall recovery of 96-97%.

**Table 1.** Weight loss after drying samples (the results are expressed as the percentage loss in weight of the samples after drying  $\pm$  standard error of three determinations).

Tissue	Sample oxidiser	Freeze-dryer	Difference
Liver	69.7 $\pm$ 1.7	69.0 $\pm$ 0.3	+ 0.7
Kidney	75.7 $\pm$ 2.1	75.7 $\pm$ 1.0	0.0
Heart	75.7 $\pm$ 0.7	75.3 $\pm$ 0.1	+ 0.4
Brain	75.4 $\pm$ 2.3	75.8 $\pm$ 2.2	- 0.4
Urine	94.3 $\pm$ 0.7	93.6 $\pm$ 0.1	+ 0.7
Faeces	62.2 $\pm$ 0.5	60.6 $\pm$ 1.3	+ 1.6
Blood	81.5 $\pm$ 1.7	82.3 $\pm$ 0.4	- 0.8
Plasma	89.6 $\pm$ 1.5	90.6 $\pm$ 0.6	- 1.0
Liver homogenate	79.2 $\pm$ 0.8	82.2 $\pm$ 1.2	- 3.0
Lung homogenate	92.7 $\pm$ 0.6	94.2 $\pm$ 0.1	- 1.5
Faecal homogenate	82.9 $\pm$ 0.7	85.1 $\pm$ 1.2	- 2.2
Water	100.7 $\pm$ 0.8	100.0 $\pm$ 0.0	+ 0.7

**Table 2.** Residual radioactivity after drying samples (the results are expressed as the percentage of the original radioactivity remaining in the samples after drying  $\pm$  standard error).

Tissue	Number of determinations	Residual radioactivity
Kidney	4	3.1 $\pm$ 0.6
Heart	4	3.6 $\pm$ 0.4
Brain	4	2.0 $\pm$ 0.2
Urine	3	3.6 $\pm$ 0.8
Faeces	4	2.9 $\pm$ 0.5
Blood	4	4.0 $\pm$ 0.6
Plasma	4	3.4 $\pm$ 0.7
Liver homogenate	4	2.8 $\pm$ 0.5
Lung homogenate	3	2.9 $\pm$ 0.4
Faecal homogenate	3	2.9 $\pm$ 0.4
Water	5	3.3 $\pm$ 0.3

**Table 3.** Recovery of tritiated water from the sample oxidiser (the results are expressed as a percentage of the total sample radioactivity).

Drying step	Combustion step	Gas effluent	Wash effluent	Total recovery
85.8	3.3	0.5	7.4	97.0
85.2	2.6	< 0.1	8.0	95.8
86.5	3.0	< 0.1	6.8	96.4

The retention efficiency of 85% is remarkably consistent. Using a wide range of biological samples, and using three different Packard 306 sample oxidisers, the retention of radioactivity in the tritium vial varied from 83–87% of the total sample radioactivity (Table 4).

Thus two methods can be employed for the estimation of tritiated water in biological samples. As an approximate measurement, the tritiated water content can be obtained by applying the 85% retention efficiency figure to the radioactivity appearing in the tritium vial during the drying step. This procedure allows a simple and rapid check for tritium exchange and is particularly suitable where the amount of tritium exchange is small. A more accurate assessment is made by a lengthier process. A sample is combusted to give total radioactive content, and a second aliquot of the same sample is first dried and then combusted. The difference in radioactivity between the two combustions is a measure of the tritiated water in the sample.

**Table 4.** Retention of tritiated water in the tritium collection vial (the results are expressed as the percentage of the total sample radioactivity retained in the tritium vial on drying  $\pm$  standard error).

Tissue	Number of determinations	Radioactivity retained
Liver	4	84.3 $\pm$ 2.5
Kidney	4	86.9 $\pm$ 1.4
Heart	4	85.9 $\pm$ 0.9
Brain	4	87.2 $\pm$ 2.8
Urine	3	83.7 $\pm$ 0.5
Faeces	4	85.7 $\pm$ 0.7
Blood	4	86.9 $\pm$ 1.2
Plasma	4	84.2 $\pm$ 0.8
Liver homogenate	4	87.3 $\pm$ 1.2
Lung homogenate	3	85.8 $\pm$ 0.9
Faecal homogenate	3	86.3 $\pm$ 1.2
Water	10	86.5 $\pm$ 1.6

## PRACTICAL USE OF METHOD

We have recently carried out a study with a tritiated compound in laboratory animals and it was first necessary to determine the degree of biological exchange. The compound was an acridine derivative, Metifex, uniformly labelled with tritium in the aromatic ring (Fig. 2) with a radiochemical purity of at least 95%. As part of the study, the compound was administered orally to male beagle dogs at a dose level of  $5 \text{ mg kg}^{-1}$ , and urine, faeces and plasma samples obtained at suitable times after dosing. The radioactive content of samples was determined either by direct counting in a suitable scintillator for urine and plasma or by combustion of aliquots of faecal homogenates.

Table 5 shows the excretion of radioactivity in urine and faeces from one male dog. The excretion of radioactivity in the urine over 148 h was no more than 4% of

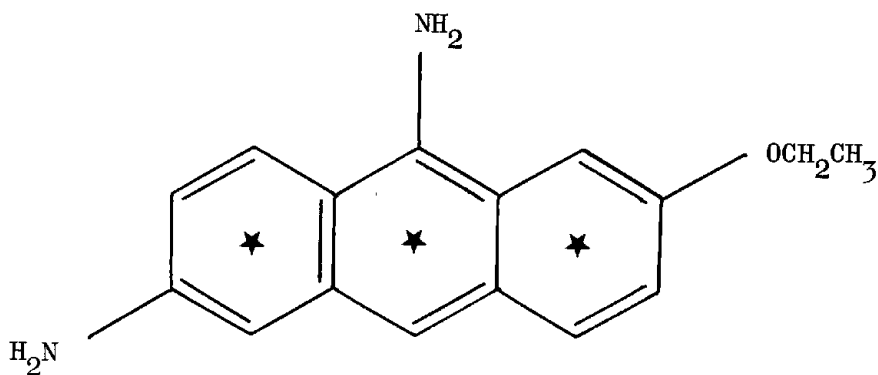


Fig. 2. 2-ethoxy-6,9-diaminoacridine (Metifex). (\* denotes aromatic ring uniformly labelled with tritium.)

Table 5. Excretion of radioactivity in urine and faeces following oral administration of  $^3\text{H}$ -Metifex to one male beagle dog (the results are expressed as the cumulative excretion of radioactivity – as a percentage of the administered dose).

Time after dosing (h)	Cumulative percentage of the dose	
	Urine	Faeces
2	0.6	ND
24	1.9	38.7
48	2.6	57.5
72	3.3	64.7
148	4.1	67.7

ND = not determined.

the dose. Faecal excretion was much greater, and after 148 h the cumulative urinary and faecal output was 72% of the dose, and the balance, including cage washings, was 76%. This poor recovery suggested that tritium exchange might be occurring, resulting in retention of tritiated water with a long biological half-life. Urine, plasma and faecal samples were therefore analysed for their tritiated water content. The results, using the 85% retention efficiency factor, are shown in Table 6. Little tritium exchange had occurred in faeces. However, urine showed an increasing amount of exchange with time from 17% at 24 h to 77% at 148 h after dosing. The majority of the radioactivity in the plasma was tritiated water. Pre-dose urine samples spiked with aliquots of the labelled dosing solution were shown to contain less than 0.3% tritiated water at zero time and after two days storage at 4 °C, indicating that spontaneous chemical exchange in urine was not occurring.

**Table 6.** Tritium exchange following oral administration of  $^3\text{H}$ -Metifex to one male beagle dog (the results are expressed as the percentage of tritiated water in the excreted radioactivity).

Time after dosing (h)	Faeces	Urine	Plasma
2	ND	3.0	ND
4	ND	ND	67.5
6	ND	ND	73.5
12	ND	ND	81.5
24	0.7	18.6	85.0
48	1.4	26.0	92.6
72	2.2	56.9	94.5
148	1.2	77.1	93.9

ND = not determined.

**Table 7.** Three methods for the estimation of tritiated water in urine (the results are expressed as the percentage of tritiated water in the excreted radioactivity – means of two determinations).

Time after dosing (h)	Sample oxidiser		Freeze-dryer
	One-stage method	Two-stage method	
2	3.0	1.1	5.0
24	18.6	18.0	16.5
48	26.0	26.6	26.3
72	56.9	52.1	53.1
148	77.1	73.1	73.7

In order to compare the methods, tritium exchange in urine was also measured by the lengthier method of two combustions, as well as by freeze-drying. Table 7 shows the percentage of urinary radioactivity which is tritiated water. This was measured using

- (i) the 85% retention efficiency factor as in Table 6;
- (ii) the two-stage method, i.e. combustion of first fresh and then dried tissue and subtracting the result to give the tritiated water content;
- (iii) freeze-drying using a conventional Virtis freeze-dryer.

The two-stage method is probably less accurate when the percentage of tritium exchange is small, but otherwise the three methods are in good agreement.

## SUMMARY

Using a simple modification to a commercially available sample oxidiser, we have been able to measure reliably both the total tritium and tritiated water content of biological samples. The method is faster and less tedious than freeze-drying, and is an extension of a normal oxidation method for preparing biological samples for liquid scintillation counting.

## REFERENCES

1. E. Rapkin, in *Liquid Scintillation Counting*, Vol. 2 (eds. M. A. Crook, P. Johnson and B. Scales), Heyden, London, 1971, pp. 61-100.
2. N. Kaartinen, *A New Oxidation Method for the Preparation of Liquid Scintillation Samples*, Packard Technical Bulletin No. 18, 1972.

## DISCUSSION

**M. J. Rance:** I would like to know if you have compared the sensitivity of this technique with more standard methods (such as enclosed metabol studies, distillation of urine etc.) for the determination of tritium exchange.

**B. R. Twite:** I have no experience of enclosed metabol studies, but the method has been compared to freeze-drying and shows comparable sensitivity.

**M. J. Rance:** Do you feel that with your method it is possible to generate reliable drug distribution data routinely in the presence of biological lability?

**B. R. Twite:** In the example used to illustrate the method, 1% tritium exchange was detectable.

**T. J. Rising:** The method is applicable to the determination of small levels of tritium exchange in the order of 1%, provided the specific activity of the original dosing solution is high enough. I believe that results so obtained could be confidently submitted to regulatory authorities.

**P. Johnson:** Could Dr. Rance elaborate his first question because I am not clear as to the relevance of studies of expired air from metabolism cages to our technique of measuring tritiated water in tissue and body fluids. Are you referring to the

amount of volatile tritium that might be exhaled? This is not measured by our technique.

**M. J. Rance:** In our laboratory, any tritiated compound showing biological lability would not be used in further studies. Nevertheless, distribution studies such as you describe could constitute a screen for exchange in the same way as we at present use total recovery studies, distillation of biological fluids, etc.

**G. Ayrey:** Is it true to say that your method assumes that the only volatile metabolic product is water? I would be interested to know if you had to check this and if there was any chance that the labelled drug was partially volatile or steam volatile under the conditions used.

**B. R. Twite:** It is, of course, absolutely essential to check the amount of tritium exchange in the dosing solution using the drying method described — indeed, this is best checked in the biological fluid in which the tritium exchange will be measured. Other volatile metabolites are evaporated from the sample, but are not necessarily retained in the tritium collection vial. The presence of other volatile metabolites will be shown by the ‘two-stage’ drying method described.

**D. Case:** What modifications, if any, have to be made to the Packard 306 in order to use the technique you describe?

**B. R. Twite:** A simple modification is required, involving the insertion of a switch in the circuitry.