A COMPARISON OF PHOSPHOR SOLUTIONS FOR COUNTING AQUEOUS SAMPLES OF STEROID HORMONES

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Abstract

The counting efficiency of phosphor solutions suitable for counting aqueous samples of non-polar and polar [^{3}H] steroids have been compared. Results show that non-polar steroids such as [^{3}H]-progesterone can be most efficiently counted as a heterogeneous system in toluene phosphor. Relative counting efficiency (sample without water = 100%) increased when water was present and was independent of water content up to 3 ml in 10 ml phosphor. Shell Sol A (methyl benzene fractions) phosphor also gave a counting efficiency independent of water content but the relative efficiency was 10-15% lower than for toluene phosphor. Emulsion systems, triton/toluene and Insta-Gel and a Dioxan based phosphor gave low counting efficiencies dependent on water content.

For the more polar steroids, e.g. [^{3}H]-cortisol, the most efficient phosphor solution was toluene phosphor with the aqueous phase 50% ammonium sulphate rather than water alone. This phosphor gave counting efficiencies independent of water content whereas all other phosphors had lower efficiencies very dependent on the amount of water present.

On the basis of cost, toluene (Shell, commercial grade) phosphor was cheap $0.73/L and as efficient as toluene (Fluka, purum) phosphor $2.0/L. Apart from Shell Sol A all other phosphors were more expensive and much less efficient.

The mechanism of the increase in relative counting efficiency seen with aqueous toluene phosphor is discussed.

Introduction

In radioimmunoassay and saturation analysis procedures used for the measurement of a wide variety of steroid hormones in biological fluids, tritium labelled steroids are widely used both as the tracer and as a recovery indicator for extraction and purification losses. Liquid Scintillation counting of aqueous samples of the tritium labelled steroid is often necessary if additional time consuming methods of preparing the samples in a non-aqueous form are to be avoided. The aim of the present study was to evaluate the efficiency and cost of a variety of phosphor solutions suitable for counting aqueous samples of steroid hormones of different polarity.

Materials and Methods

Tritiated steroids, [1,2-^{3}H]-aldosterone, [1,2-^{3}H]-cortisol [1,2-^{3}H]-corticosterone, [1,2-^{3}H]-deoxycorticosterone, [6,7-^{3}H]-17β-oestradiol, [6,7-^{3}H]-oestrone, [1,2-^{3}H]-testosterone and [1,2-^{3}H]-progesterone were obtained from either the Radiochemical Centre, Amersham, U.K. or New England Nuclear Corporation, U.S.A. All were of specific activity 30-50 Ci/mM and purified by paper partition chromatography before use. [^{3}H]-steroids were stored in ethanol at -4^{o}C. Non-radioactive steroids (Ikapharm) were stored as a 1 mg/ml solution in ethanol.

Solvents used in preparation of phosphor solutions, toluene (Fluka, purum) and dioxan (Fluka, puriss), were used without purification. Toluene (Shell, commercial grade) and Shell Sol A (methylated benzene fractions) were filtered prior to use. Triton X 100, Insta-Gel, PPO and dimethyl POPOP were obtained from Packard Instrument Co. All phosphor solutions, except Insta-Gel and the dioxan based phosphor, contained 4g/L PPO and 40 mg/L dimethyl POPOP. The dioxan based phosphor contained 100 ml ethoxyethanol, 20 ml ethylene glycol, 8g PPO, 600 mg dimethyl POPOP, 150 g napthalene and dioxan to make 1 litre (1). All samples were prepared in glass counting vials (Packard Instrument Co.) and counted in a Packard Model 314 EX, 3330 or 3375 liquid scintillation spectrometer. Samples were prepared in triplicate by addition of non-radioactive steroid (50 μg) and at least 50,000 dpm of [^{3}H] - steroid to each counting vial. After removing the ethanol by drying, 0.5 to 3 ml of water was added followed by 10 ml of phosphor solution. Samples were counted for at least 3 x 10 mins. after samples had been thoroughly mixed and allowed to equilibrate at the temperature of the counting chamber for 10-12 hr.

Results

Samples of all [^{3}H] - steroids to be compared were prepared in: a) toluene (Fluka) phosphor, b) dioxan based phosphor, c) triton X-100/toluene phosphor (2/1 v/v). Samples were counted in a Packard 314 EX at -10^{o}C at 25% tritium efficiency and in a Packard 3375 at 0^{o}C or 20^{o}C at 49% tritium efficiency. Results for the toluene (Fluka) phosphor are shown on Table 1. All results are shown as relative efficiency, the sample without water being expressed at 100%.

TABLE I

Relative efficiency of samples counted in toluene (Fluka) phosphor containing 0.5-3.0 ml water. All samples counted in a Packard 3375 at 0^{o}C at a tritium efficiency of 49%. Sample without water expressed as 100%.

STEROID	WATER VOLUME (ml)				
	0.5	1.0	1.5	2.0	3.0
[^{3}H]- Aldosterone	74.7	60.1	50.4	43.3	-
[^{3}H]- Corticosterone	101.3	100.7	98.9	99.3	-
[^{3}H]- Cortisol	68.4	52.2	50.9	34.3	27.1
[^{3}H]- DOC	104.7	104.7	105.1	105.8	-
[3H]- Oestradiol	100.6	101.4	101.8	102.0	-
[^{3}H]- Oestrone	99.4	101.1	101.7	101.8	-
[^{3}H]- Progesterone	102.7	103.2	102.6	101.9	103.9
[^{3}H]- Testosterone	104.0	104.3	105.1	105.3	-

Similar results were obtained when the above examples were counted at either -10°C in a Packard 314 EX at a tritium efficiency of 25% when the aqueous layer was frozen or at 6°C in a Packard 3330 at a tritium efficiency of 65%.

Table I shows that counting efficiency of the most polar of the steroids, [^{3}H]-aldosterone and [^{3}H]-cortisol, fell with increasing water content of the samples. However, for the other non-polar steroids, counting efficiency did not change with water content and in most cases was greater than if no water was present. The degree of increase in relative efficiency appeared to be a function of a particular steroid. In the remainder of the studies to be reported, [^{3}H]-cortisol and [^{3}H]-progesterone have been used as examples of the polar and non-polar steroid types.

A comparison of the relative efficiency (100% is for the water free toluene (Fluka) phosphor sample) of three different phosphor solutions for aqueous samples of [^{3}H]-cortisol and [^{3}H]-progesterone are shown on Figure 1. All samples were counted at 0°C in a Packard 3375 at a tritium efficiency of 49%.

For [^{3}H]-progesterone, the dioxan based phosphor and the triton/toluene phosphor both gave much lower counting efficiencies when compared with the toluene phosphor. Efficiencies were much lower even without water present and showed a considerable decrease with increasing water content. With the toluene phosphor, relative counting efficiency was very high and independent of water content (Table I).

On the other hand, for [^{3}H]-cortisol the results were similar for all three phosphor solutions. All showed a marked reduction in counting efficiency with increasing water content. Figure 1 also shows that recounting the dioxan phosphor at 20°C to prevent freezing increased efficiencies for both [^{3}H]-progesterone and [^{3}H]-cortisol compared with those obtained at 0°C.

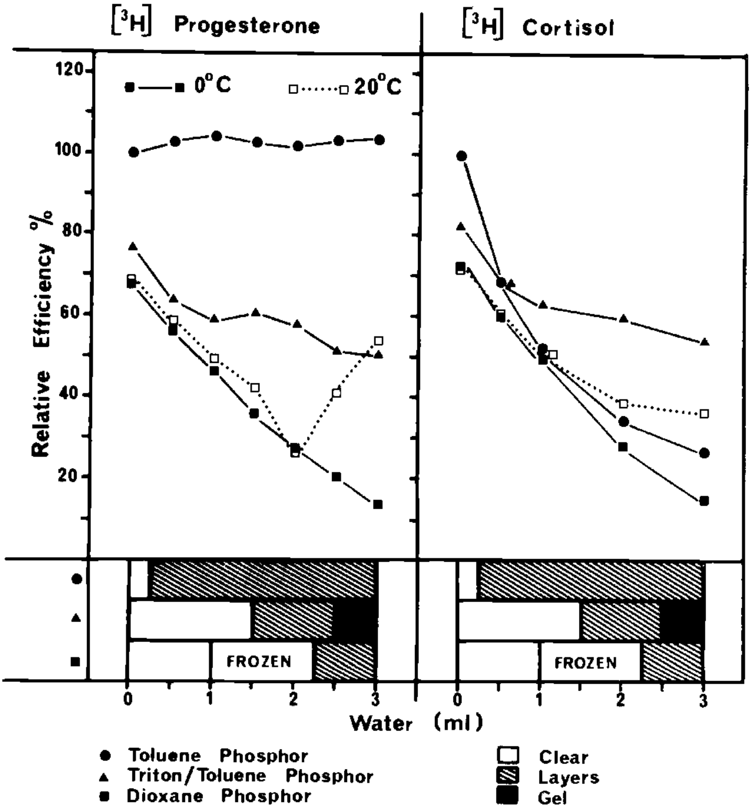

Figure 1. A comparison of the relative efficiency of toluene phosphor, triton X-100/toluene phosphor and dioxan based phosphor for counting aqueous samples of [^{3}H] -cortisol. Samples were counted in a Packard 3375 at 0^{o}C and 20^{o}C at a tritium efficiency of 49%.

TABLE II

Relative efficiency of [^{3}H]-progesterone samples counted in toluene phosphor containing 0-10 ml water. Samples counted in a Packard 3375 at 0^{o}C at 49% tritium efficiency.

	WATER VOLUME (ml)						
	0	2.0	3.0	4.0	5.0	7.0	10.0
RELATIVE EFFICIENCY	100	104.2	103.6	104.2	103.5	101.6	96.7

The effect of increasing the water content up to 10 ml in 10 ml of toluene phosphor for [^{3}H]-progesterone is shown on Table II. The fall in efficiency observed with the larger water volumes may be due to poor positioning of the toluene phase of the phosphor in the counting chamber.

Availability of low cost commercial grade toluene (Shell) and a methyl benzene based solvent (Shell Sol A) have enabled these to be compared with the purum grade toluene (Fluka). Results for samples containing either [^{3}H]-progesterone or [^{3}H]-cortisol are shown on Figure 2. A comparison is also made with Insta-Gel (Packard) and with samples containing 50% ammonium sulphate rather than water. Samples were counted at 6^{o}C in a Packard 3330 with a tritium efficiency of 65%. Relative efficiency is expressed as 100% for the water free toluene (Fluka) phosphor.

[^{3}H]-progesterone had similar counting efficiencies and increase in relative counting efficiency in the presence of water in both the Fluka and Shell toluene phosphor solutions. However, when counted in Shell Sol A,the relative efficiency was much lower and averaged 12% less than with toluene phosphor. Insta-Gel caused considerable loss in counting efficiency even in the absence of water and efficiency was dependent on the water content of the samples. Instability of the solution was observed in

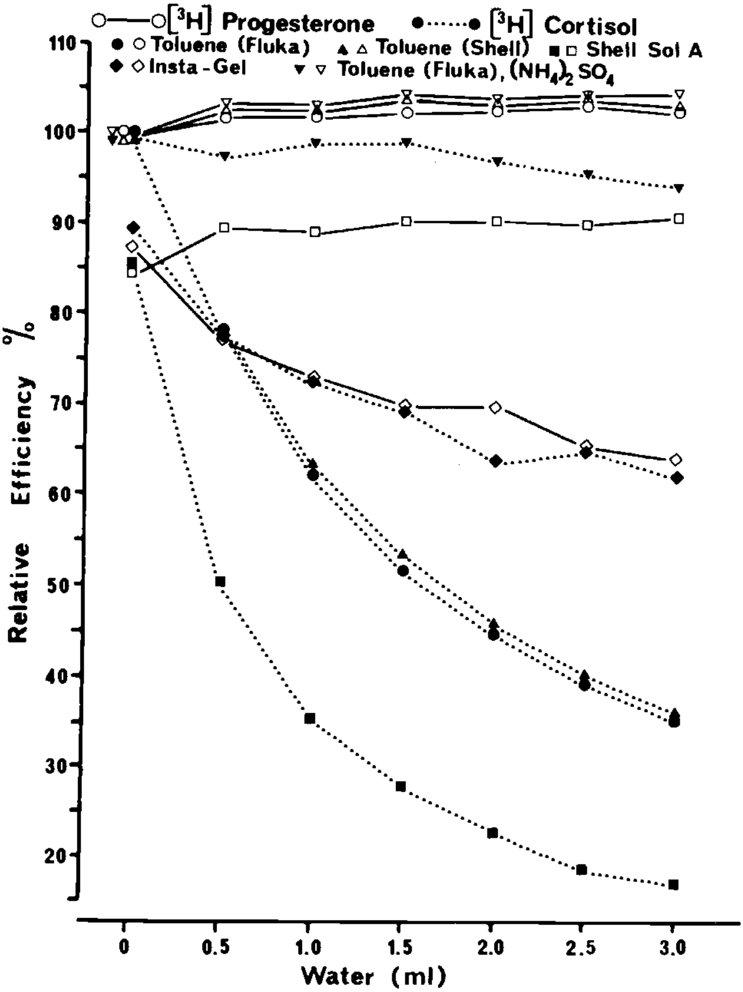

Figure 2. A comparison of the relative efficiency of various phosphor solutions used to count aqueous samples of [^{3}H]-progesterone and [^{3}H]-cortisol. Samples were counted in a Packard 3330 at 6^{o}C at a tritium efficiency of 65%.

the sample containing 2 ml water. [^{3}H]-progesterone counted in toluene and containing 50% ammonium sulphate rather than water had a similar counting efficiency to toluene phosphor containing water alone.

For the more polar steroid, [^{3}H]-cortisol, both the Fluka and Shell toluene phosphors showed the water dependent fall in efficiency previously observed (Figure 1). Shell Sol A had a relative counting efficiency 20-25% lower than for toluene and showed a similar loss in efficiency with addition of water. Insta-Gel produced a relative efficiency - water content curve similar to that seen with [^{3}H]-progesterone. The most interesting result observed was when 50% ammonium sulphate was added to the [^{3}H]-cortisol samples rather than water. Relative counting efficiency increased markedly over that seen with water alone and was independent of water content with up to 1.5 ml water. Efficiency then fell slowly with increasing water content to 95% with 3.0 ml of water.

Table III combines and summarises the results for each of the phosphor solutions examined.

Results are expressed as relative counting efficiency, 100% is for toluene (Fluka) sample without water. Table III shows that for non-polar steroids, e.g. [^{3}H]-progesterone, there is an increase in counting efficiency in toluene phosphor and in Shell Sol A when water is added. Once water has been added efficiency is independent of water content. For polar steroids, e.g. [^{3}H]-cortisol, 50% ammonium sulphate added to the toluene phosphor solution increases relative counting efficiency.

TABLE III

Relative counting efficiency for various phosphor solutions used to count aqueous samples of [^{3}H]-progesterone (A) and [^{3}H]-cortisol (B)

(A) [^{3}H]-PROGESTERONE

PHOSPHOR	WATER VOLUME (ml)			
	0	1.0	2.0	3.0
1. Toluene (Fluka)	100	103	102	104
2. Toluene (Shell	100	103	103	104
3. Shell Sol A	85	89	91	91
4. Dioxan based	68	46	27	14
5. Triton/Toluene	77	59	58	50
6. Insta-Gel	87	73	70	64
7. Toluene & Amm.Sulphate	100	103	104	105

(B) [^{3}H]-CORTISOL

PHOSPHOR	WATER VOLUME (ml)			
	0	1.0	2.0	3.0
1. Toluene (Fluka)	100	62	45	35
2. Toluene (Shell)	100	64	46	36
3. Shell Sol A	85	35	23	17
4. Dioxan based	73	50	28	15
5. Triton/Toluene	82	63	60	54
6. Insta-Gel	89	73	64	62
7. Toluene & Amm.Sulphate	99	99	97	95

Another factor examined in this study was the cost of the various phosphor solutions. Table IV shows the cost of both the solvent component and the total phosphor solution. Brays Solution (2) is shown for comparison. Prices were those available in Australia and have been converted to U.S.$.

TABLE IV

PHOSPHOR SOLUTIONS FOR AQUEOUS SOLUTIONS*

SOLVENT	COST/L $	PHOSPHOR COST/L	RELATIVE COST
1. Toluene (Fluka)	1.40	2.00	2.7
2. Toluene (Shell)	0.13	0.73	1.0
3. Dioxan (Fluka) Blend (1)	6.27	10.57	14.5
4. Shell Sol A	0.14	0.74	1.0
5. Triton/Toluene (2/1)	1.40	1.79	2.4
6. Insta-Gel	-	8.75	12.0
7. Brays Solution (2)	-	6.34	8.7

* All solutions except (3) and (6) contain 4g/L PPO ($0.85/L) and 0.04 g/L dimethyl POPOP ($0.05/L). All costs based on Australian $ converted to U.S.$.

Also, they are the lowest available for the particular product of a stated quality. The relative cost shown on Table IV is based on the cheapest toluene (Shell) phosphor. When the cost factor is taken into account with the counting efficiency data previously presented in Figures 1 and 2 and Table III it is clear that for non-polar steroids in aqueous solution the Shell toluene solution is the phosphor of choice. It is cheap and gives high counting efficiency independent of water content. For the more polar steroids addition of ammonium sulphate to the aqueous phase of toluene (Shell) phosphor provides a cheap scintillation solution giving a high relative counting efficiency independent of the amount of water present.

The reproducibility of counting a [^{3}H]-progesterone sample in 2 ml of water in toluene phosphor has been examined. Ten replicate samples containing 3.5 x 10^5 dpm were prepared and each counted 7 times for 10 mins in a Packard 3330. The mean between sample variability was 0.2% and that between individual counts of the same sample 0.6%. A similar check on reproducibility was carried out for [^{3}H]-cortisol in samples containing 2 ml of 50% ammonium sulphate. Variability was similar to that found for [^{3}H]-progesterone.

To further investigate the increase in counting efficiency caused by the presence of water in the toluene phosphor, the effect of adding water to phosphor containing [^{3}H]-toluene was examined (Table V).

An increase in efficiency was observed with as little as 0.2 ml of water in the 10 ml of [^{3}H]-toluene phosphor. A small increase in relative counting efficiency was also observed for both [^{3}H]-progesterone (102.5%) and [^{3}H]-cortisol (101.3%) if water saturated toluene rather than anhydrous toluene was used to prepare the phosphor solution.

TABLE V

The effect of water on the relative counting efficiency of [^{3}H]-toluene phosphor. Samples were counted in a Packard 3375 at 0°C at a tritium efficiency of 49%.

	WATER VOLUME (ml)				
RELATIVE COUNTING EFFICIENCY	0	0.2	1.0	2.0	3.0
	100	103	105	107	106

Discussion

The most interesting finding of these studies was the increase in relative counting efficiency observed for the non-polar steroids when they were counted in aqueous toluene phosphor. Further, this increase in efficiency appeared to be relatively independent of water content up to 30%. The high counting efficiency of non-polar steroids can probably be explained on the basis of the high extraction of these steroids from the aqueous phase into the toluene. However, this does not explain the increase over 100% seen in many of the experiments reported in this paper. This will be discussed in detail later. It is not possible to explain the difference in behaviour between the individual non-polar steroids reported on Table I without more detailed studies. The lower counting efficiency, dependent on water content, observed for the more polar steroids is presumably due to their poor partitioning from the water into the toluene phase. Addition of ammonium sulphate to the aqueous phase increases the extraction of the [^{3}H]-cortisol into the toluene and results in greater counting efficiency. A number of explanations of the 4-5% increase in relative counting efficiency seen when water is added to the toluene phosphor have been considered.

Firstly, it is unlikely that adsorption of the [^{3}H] steroid to the wall of the vial, which is removed by addition of water, has occurred since at least 50 μg of non-radioactive steroid is added to each vial as carrier. Secondly, the samples without water could be quenched and when water is added the quenching material partitions into the water phase with a resultant increase in efficiency. Although this could explain the observed results if a narrow counting window was used it is unlikely to have been responsible when the spectrometer was set to give maximum tritium efficiency. Thirdly, if the counter had been not set up properly at balance point for tritium,addition of water may have quenched the samples and shifted the spectra back into the window resulting in an apparent increase in counting efficiency. This would also be unlikely for a counter set up for maximum tritium efficiency. The most likely explanation is that it is due to an optical effect within the counting vial; possibly due to internal reflection at the toluene-water interface. Some of the photons which are normally lost through the base of the glass vial are reflected and eventually are observed by the photomultiplier tubes. A similar effect has been observed by Gordon and Curtis (3); they reported an increase in efficiency in samples containing optically diffusing white materials or surfaces. The enhanced efficiency they suggested resulted from a reduction in the amount of light lost through total internal reflection.

Although it is not easy to measure actual counting efficiency in such a heterogenous counting system as is described in this paper, this is not so important in saturation analysis where standards and samples are counted in the same way.

In summary, a cheap, efficient phosphor solution suitable for counting aqueous samples of [^{3}H]-steroid hormones is described. A feature of the toluene phosphor system for non-polar steroids is the independence of counting efficiency on water content.

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