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RADIOTRACER INVESTIGATION OF A QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC SEPARATION

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SUMMARY

Radioactive labelling has been shown to be a powerful technique for investigating quantitative thin-layer chromatography. The dispensing of small volumes by several appliances has been shown to be precise but biased to the extent of several percent by "creep back" of the dispensed solution on the needle of the syringe. Losses in the thin-layer chromatographic separation and recovery of phthalimide once the solution had been dispensed totalled approximately 3%. These losses were attributed to a residue at the site of application to the plate, underestimating the size of the developed spot and the failure of a fraction of the phthalimide to elute.

INTRODUCTION

Thin-layer chromatographic (TLC) and paper chromatographic separation and quantitative analysis are often unsatisfactory with relative precisions of up to 10% being quoted[1]. One of the major problems in basic research into such quantitative separations is the very small mass which must be employed on the chromatogram itself and the consequent difficulty of detecting the loss of a few percent of this. Thus if 5 μg is taken as a typical loading mass one would need to detect 1% of this, *i.e.*, 50 ng. This will normally require analytical apparatus of some sophistication. However if radiotracers are used the detection apparatus may be cheap and the method still be extremely sensitive.

^{14}C is obviously the most important label to consider and a typical minimum figure for its specific activity is 50 mCi/mmole. Therefore if the molecular weight is 100 and the detection efficiency is 10%, it is possible to detect a 50-ng mass with a coefficient of variation from randomness of decay for the sample of 1% in approximately 100 sec counting time. With a counting period of 5000 sec the mass detectable with this error is reduced to 1 ng. Thus the randomness of decay should not provide an error of any consequence.

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Specific activities for other radionuclides are generally higher than that for ^{14}C thus giving even greater ease of detection if required. For instance tritiated compounds can have specific activities up to 50 Ci/mmole and ^{32}P is available as orthophosphate at 100 Ci/mg (ref. 2). Other radiochemical errors can of course occur, but these will vary with the properties of the ionising radiation produced. Errors of absorption or self absorption should not occur with strong β- or γ-emitters although they could occur with weak β-emitters such as ^{3}H, ^{14}C or ^{35}S. Quenching in liquid scintillation counting of weak beta emitters can occur so alternative methods of counting may be preferred.

Quantitative chromatographic separations can be divided into three stages: (1) application, (2) development and possibly (3) transfer and elution. Stage 3 may be avoided by direct *in situ* measurement of the separated compound on the chromatogram by, for example, visual comparison, densitometric, spectrophotometric, fluorescence or radiochemical methods[4,5]. This removes errors from elution and reduces problems from blanks[6,7]. If the elution yield is at a constant value less than 100% it is of course possible to correct for the loss[8]. Radiotracers are well suited to yield determinations of this type as unambiguous measurement of eluant and residue on the adsorbent are possible.

For a similar reason radiotracers may be used in investigations of the precision and accuracy of application to the chromatogram as the quantity of substance delivered as well as the quantity of residue on the dispensing device can be measured directly. Choice of dispensing technique, *e.g.*, streaking or spotting, and dispensing appliance can have a critical effect[9,10].

The developed chromatogram may be examined by any of the direct in situ techniques mentioned above[4,5]. Radioactive spots may be located by a typical scanning system which moves the TLC plate relative to the detector or very ingeniously by a static reader which has a detector the length of the chromatogram which records the location of the active site by the delay time in the collection of the pulse (Numelec, Chromolec 101). Visual images of the radiochromatogram can be obtained when a spark chamber and polaroid films are used (Panax, Beta-graph). However, if none of these moderately expensive pieces of apparatus are available, it will be necessary to use photographic film and allow time for the development of suitable autoradiographs. Time will again be the variable parameter which allows suitable sensitivity to be obtained.

The separation of phthalimide has been chosen for the investigation because of its importance as a degradation product of the fungicide Folpet, [N-(trichloromethylthio)phthalimide][11,12].

EXPERIMENTAL

All radioactivity measurements except for one series were carried out with an Isotopes Developments 723 scintillation drawer unit containing a plastic scintillation phosphor (Nuclear Enterprises 102A) coupled to a photomultiplier (E.M.I. 6097B). The counter was a Nuclear Enterprises ST5. Optimum counting conditions[13] were found to be an E.H.T. of 1600 V and a threshold of 0.1. This provided a 2π counting efficiency of 78% for ^{14}C with a background count rate of approximately 8 counts per sec. A χ-squared test[14] was carried out on the counter at the start of

the work and found to be satisfactory. Daily checks on the efficiency of the detector were carried out using a standard ^{14}C source.

The measurement of the radioactivity on the surface of the syringe was carried out with an Isotope Developments lead castle 710 containing a Mullard Geiger–Müller tube MX 123 linked to the same counter as above. The E.H.T. was 600 V providing a 2π efficiency for ^{14}C of 21% and a background count rate of approximately 1 count per sec. Readings were corrected for a dead-time of 400 μsec.

Radioactive phthalimide and Folpet were prepared from ^{14}C-phthalic anhydride obtained from the Radiochemical Centre (Amersham, Great Britain). Phthalimide was dispensed in acetone solution except when indicated otherwise and in the testing of the precision of the Microcaps when an aqueous solution was used. Folpet was used in a chloroform solution. Dimpled planchets of overall diameter 2.6 cm and depth 1.75 mm were obtained from A. Gallenkamp (London, Great Britain).

TLC plates (5 × 20 cm) with 0.25 mm silica gel were obtained from Scientific Industries (Mineola, N.Y., U.S.A.). These were developed with 1% methanol in chloroform solution at room temperature (20°) in an ascending direction. Sections of silica gel were transferred to a 1-cm diameter sintered disc microfilter of porosity 4 (James A. Jobling–Corning, Stone, Great Britain) through a 9 × 1 cm I.D. glass tube which was bent at right angles and drawn out to provide a nozzle at the end[15]. Silica gel lifted with a micro-spatula from the TLC plate was immediately sucked through the nozzle onto the filter by application of a vacuum.

Application of aliquots was via a 10-μl BB constriction pipette (Horwell, (London, Great Britain), an Agla micrometer syringe (Burroughs Welcome, London, Great Britain), a 10-μl Terumo microlitre syringe (Shandon Southern Instruments, Camberley, Great Britain; type UMSG-10) and 10-μl Microcaps (10-μl disposable micropipettes; Drummond Scientific, Broomall, Pa., U.S.A.).

RESULTS AND DISCUSSION

Radioactive counting

^{14}C was counted using a solid plastic phosphor scintillation detector rather than the conventional liquid scintillation counter because dispensing onto a planchet rather than into a liquid is more similar to a TLC application. An additional advantage was that there can be no quenching errors. However errors can arise from absorption of the weak β-radiation by the phthalimide itself *i.e.*, self-absorption[16]; this will obviously be less significant if the solid is spread as thinly as possible on the planchet. The planchets were therefore washed with a Tepol solution and dried before use; the surfactant allowed even spreading of the activity so that there was no self-absorption.

A second possible error arises from the irreproducible positioning of the planchet on its tray relative to the plastic phosphor. A reduction of up to 8.7% of the count rate was found to occur if the source was not placed centrally. A metal ring made to hold the planchets was therefore used to position the source reproducibly and centrally.

A further cause of error independent of the type of detector arises from the statistical nature of radioactive decay. The standard deviation of any count is equal to the square root of the number of counts[2]. Thus on many occasions one aims to

collect 10,000 counts giving a coefficient of variation of 1%. However when one is seeking small differences in performance, the counting error should be small, compared to the error in the performance. Thus 400,000 counts were usually collected for each sample, giving a coefficient of variation of the count rate of 0.16%.

Total yield

Five 10-μl aliquots of active phthalimide solution were applied to a TLC plate using a Terumo microlitre syringe held in a near horizontal position in two lots of 5 μl each. After air drying for several minutes the plate was left to develop for 30 min. After removal and air drying the phthalimide spot was located under a 254-nm wavelength lamp, marked and transferred by vacuum suction onto a filter. The active phthalimide was then eluted from the silica gel with 2 ml chloroform which was then transferred in aliquots to a planchet for evaporation under an infrared drying lamp. The planchet was then counted and the result compared to the activity of 10 μl of the original solution. It can be seen (Table I) that the yield obtained was always less than 100%, the average loss being 3.2%.

TABLE I

YIELD OF PHTHALIMIDE AFTER TLC SEPARATION, TRANSFER AND ELUTION

Sample No.	*Yield (%)*	*Residue (%)*
1	96.2	0.34
2	96.8	0.26
3	96.2	0.41
4	97.6	0.32
5	97.2	0.42
Average	96.8	0.35

In the separations carried out above care was taken to ensure that the phthalimide was in contact with the silica gel for the shortest possible time in order to minimize any possible decomposition of the organic compound. However one aliquot was purposely left on the plate after development for over 6 h before removal. The subsequent recovery was found to be 97%; this was in agreement with the other yields obtained indicating that the phthalimide did not decompose into material insoluble in the eluting solvent under these conditions.

Application

The precision of a constriction pipette, an Agla syringe, a microlitre syringe and Microcaps were compared by dispensing 10-μl aliquots of active phthalimide solution onto planchets, evaporating off the solvent and counting. A straight delivery of the 10 μl was made in each case with the appliance held vertically except for the Agla syringe when the rotating method necessitated two 5-μl aliquots. Ten replicates were taken with each appliance. The results (Table II) indicate considerable error in each case although the Agla syringe was clearly the most precise. However this is not an absolute order of precision as there must be an operator factor involved.

The precision of an operation is, however, in many cases less important than its absolute accuracy. A possible error in application is due to "creep back" of active

TABLE II

PRECISION OF REPLICATE DISPENSING APPLIANCES

Appliance	*Coefficient of variation (%)*
Constriction pipette	1.64
Agla syringe	0.56
Microcaps	1.35
Terumo microlitre syringe	1.03

solution on the outside of the needle[9]. This was investigated in the case of the microlitre syringe by measuring the activity on the exterior of the needle after it had dispensed 10-μl aliquots. Three techniques of dispensing were used: (1) a single 10-μl volume ejected straight from a syringe held vertically; (2) a slower ejection allowing evaporation of single drops as is often used in TLC applications; (3) two approximately 5-μl aliquots ejected with the syringe held near horizontally with the slanted hole of the needle facing downward.

After delivering the volume as above, the dead volume of solution was withdrawn into the syringe barrel and the syringe taped to a plastic holder so that the needle was positioned immediately under the mica window of a Geiger–Müller tube. This detector was used because of the impossibility of physically placing the syringe inside the light-tight box of the scintillation detector. The metal of the needle was of sufficient thickness to shield the detector from radioactive material deposited on the inside or on the opposite side of the needle to the detector. The energy of the β-particle from ^{14}C is 0.155 MeV indicating a range in steel of approximately 0.03 mm. The needle was counted twice; once with the slanted hole facing downward (upper side counted) and second with it facing upward (lower side counted).

The results (Table III) which were obtained for three different solvents namely acetone, chloroform and toluene indicated that a considerable percentage of the 10-μl solution must have "crept back" up the needle with subsequent evaporation and deposition of the radioactive solute. This loss was clearly greatest when the volume was dispensed slowly as is usually the case with TLC applications. There was no obvious advantage in changing the solvent from acetone (b.p. 56.2°) to chloroform (b.p. 61.7°) or toluene (b.p. 110.6°), so factors other than ease of evaporation such as interfacial tension must be involved.

It should be noted in the total yield experiments above that the yield was determined by comparison of the recovery obtained with an aliquot dispensed di-

TABLE III

VARIATION OF PERCENTAGE OF MICROLITRE SYRINGE VOLUME LEFT AS RESIDUE ON UPPER OR LOWER SIDES OF NEEDLE EXTERIOR WITH DIFFERENT SOLVENTS

Mode of application	*Percentage left as residue*					
	Acetone		*Chloroform*		*Toluene*	
	Upper	*Lower*	*Upper*	*Lower*	*Upper*	*Lower*
(1) Straight 10 μl	1.8	1.0	0.9	0.4	1.7	1.2
(2) Single drops	4.5	3.8	3.3	1.8	2.6	1.3
(3) 2 × 5 μl	1.4	0.4	1.3	1.0	2.6	1.2

rectly onto a planchet. There was thus probably a loss due to creep back in the dispensing of this reference activity (approximately 1.0%) which should be added to the overall loss. In addition it should be noted that the possible losses from application could easily outweight all the other losses in the system added together. This conclusion is in agreement with that of other workers[9].

Results such as those above can be particularly insidious, since the initial tests on the appliances indicated high reproducibility in their delivery. However the volume actually delivered will clearly vary with the appliance, the rate of ejection, the angle of delivery and the nature of the solution.

Development

Active material can be located very easily on the developed TLC plate by means of autoradiography[17]. The dried TLC plate can be held next to a photographic film for a variable length of time to give the required sensitivity of detection and the technique may be made quantitative by examining the film with an optical densitometer if necessary. The R_F value of the phthalimide was found to be 0.48 by autoradiography. That of Folpet, which is the principal compound from which it must be separated, was determined in a similar way using labelled Folpet and found to be 0.72. A clear separation could therefore be obtained.

Autoradiographs of phthalimide chromatograms also indicated that some radioactivity remained at the site of application. This could have been due to a radioactive impurity in the phthalimide, decomposition of the phthalimide or reaction of the phthalimide with the adsorbent. Visual comparison with autoradiographs of standard active masses indicated that the residual activity was approximately 1% of that originally applied to the plate.

Elution

In the first experiment described in which the total yield of phthalimide was determined after development, transfer and elution, the residual silica gel after elution[18] was removed from the filter funnel, placed on a planchet and counted. The activity determined is expressed as a percentage of the activity which had been eluted to give the percentage residue (Table I). The average percentage residue of 0.35% is an underestimate of the activity remaining because the efficiency of the detection is considerably impaired by absorption of the beta radiation by the silica gel.

In addition the silica gel remaining on one of the plates used in this experiment was removed and counted. The activity found in the area between the spot and the solvent front was equivalent to 0.40%, whilst that between the spot and just below the application point was equivalent to 1.3%. In a second test the area from 3 mm below the original developed spot up to the spot itself yielded 0.58%, whilst the small area at the point of application yielded 0.64%. It is therefore clear that the ultraviolet fluorescence method used to locate the phthalimide spot underestimated its area. Also again there is evidence of either an active impurity in the phthalimide or of decomposition or reaction on spotting. Anyhow 100% recovery from a strong adsorbent such as silica gel is not expected[19].

The efficiency of chloroform, acetone and methanol as eluting solvents were compared. Active phthalimide spots on silica gel were transferred to a filter funnel in the usual manner. Aliquots (1 ml) of the solvent were then collected after they

had passed through the silica gel, evaporated down and counted. The activity of each ml is expressed as a percentage of the total activity removed by 4 ml solvent (Table IV). It can be seen that there is little point in eluting with more than 2 ml solvent despite the fact that it has been shown that there is some residual activity on the silica gel.

TABLE IV

EVALUATION OF ELUTING SOLVENTS

Volume (ml)	*Activity eluted (%)*		
	Chloroform	*Acetone*	*Methanol*
1	99.5	99.5	99.6
2	0.46	0.33	0.33
3	0.08	0.12	0.043
4	0.009	0.032	0.000

CONCLUSIONS

Initial experiments indicated that the average loss in the quantitative separation and elution of phthalimide was 3.2%. However subsequent experiments indicated that the true loss was probably greater than this because there was an additional loss due to "creep back" on the needle of the dispensing microlitre syringe.

The major loss once the phthalimide had been applied to the plate was from underestimating the developed spot area from its ultraviolet fluorescence. This accounted for nearly 1% of the loss. There was also a loss (>0.64%) due to a residue at the point of application. All the eluting solvents tested were efficient and yet a residue remained on the silica gel equivalent to a loss of more than 0.35%. Thus it is thought that the primary sources of error in the quantitative separation of this organic compound have been determined very easily by the use of radiotracer techniques.

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