SOURCES OF ERROR IN QUANTITATIVE PAPER AND THIN LAYER CHROMATOGRAPHY

I. PRODUCTION OF THE INITIAL SPOTS

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SUMMARY

A study of the errors involved in measuring small volumes of solution to form the initial spots of a chromatogram has been made. Radioactive solutes were used so that quantities could be measured conveniently and rapidly as radioactivities. Errors of up to 20 % were found even with experienced workers using conventional syringes or pipettes. Investigation of this surprisingly large source of error showed it was mainly due to "creep back" up the outside of the needle during repetitive delivery and "capillation" from within the needle when touching the surface of the adsorbent. A machine has been devised to overcome these defects; using it the errors of replicate delivery of small volumes have been reduced to about $\pm$ 4 %.

The use of thin layer and paper chromatography for quantitative estimations is extremely attractive owing to its simplicity, the small quantities of material involved and the possibility that several substances can be estimated simultaneously. In principle the method should be accurate since, in identical circumstances, the quantity of substance in the final spot should always be in a fixed ratio to the quantity applied in the initial or starting spot. If the elution or staining and measuring etc. of the final spot is also carried out in identical conditions then the ultimate measurement (optical density, spot area, densitometer reading) should accurately represent the amount of substance originally applied. In practice, however, it is extremely difficult to assure identical conditions for every chromatographic run, and to assist this various precautions are recommended. The cleanliness of the paper should be ensured by abstracting sheets from properly sealed boxes and using gloves when handling sheets[1]. The initial spots should be of constant area[2] and as small as possible[3], the paper equilibrated overnight[3], and the chromatographic run carried out at constant temperature[4] in an undisturbed tank[5] containing a "critical solvent volume" of 1 %[6]. Drying and spraying should also be carried out in controlled conditions[1]. To meet certain conditions which are not under the control of the worker, such as variations within and between sheets of paper, some workers recommend the use of standard and control solutions for every determination on the basis of the "four point bioassay" technique[4,7,8].

The success of the precautions taken by workers will be reflected in the reproduci-

bility of the method. Unfortunately not all workers test their reproducibility by proper statistical analysis of an adequate number of replicates. One well recognised statistic is the coefficient of variation (standard deviation of individual results expressed as the percentage of the mean). If the individual results form a normal distribution then 95 % ($P = 0.95$) of the results will fall within a range of $\pm$ twice the coefficient of variation. When insufficient replicates have been made it may not be possible to check whether the distribution is normal or to estimate the true standard deviation; the variation may therefore be three times the coefficient of variation, or even more. A coefficient of variation of 5 % will therefore indicate that individual results will vary ($P = 0.95$) $\pm$ 10 to 15 % of the mean. Obviously if the figures quoted by workers are the *means* of several results, the variation will be less than that for individual results. This "standard error of the mean" can be obtained by dividing the variation of individual results by the square root of the number of results used in arriving at the mean.

Some examples of published variations for quantitative chromatographic methods are: FAIRBAIRN AND WASSEL[4], coefficient of variation 6 to 6.8 %; MCEVOY-BOWE AND LUGG[9], coefficient of variation (calculated from their standard error of the mean of triplicate assays) 7.7 %; RÖMISCH[10], coefficient of variation (estimated from his figures) 8 to 16 %; GENEST AND FARMILO[11], $\pm$ 4 %. BUSH[1] quotes maximum variations of $\pm$ 5 % or $\pm$ 7 % for experienced workers; presumably these would be equivalent to coefficients of variation of about 3 %.

Recently we have attempted to devise a quantitative method for the estimation of opium alkaloids based on densitometer readings of the final spots. The precautions recommended by other workers as well as those based on our own experience were rigidly adhered to but there was no real improvement in accuracy over the figures already quoted. The coefficient of variation for the results for morphine was $\pm$ 5.2 %, for codeine $\pm$ 6.1 % and for thebaine $\pm$ 6.7 %. We therefore decided to extend the search for sources of variation to two of the earliest stages in the chromatographic procedure, namely the delivery of known volumes of solution to form the initial spot and secondly, the translocation of the molecules to the final spot during the chromatographic run. The latter item is the subject of our second paper.

EXPERIMENTAL

In view of the large number of analyses and small quantities involved, it was decided to use radioactive substances so that quantities could be measured as radioactivities[12]. For this purpose D-glucose-^{14}C(U) (sp.act. 3.9 mC/mmole) L-tyrosine-^{14}C(U) (243 mC/mmole) and morphine-2-T (0.15 mC/mmole) were used; radioactivities were determined in a Packard Scintillation Counter (Tricarb Model 574) using the following phosphor:

Naphthalene	80 g
PPO	5 g
Dimethyl POPOP	0.05 g
Xylene	390 ml
1,4-Dioxan	390 ml
Ethanol	235 ml

Preliminary experiments were necessary in order to determine the instrumental errors involved. Furthermore, as we intended delivering the solutions of radioactive

substances on to paper or thin layer chromatograms and then transferring the paper or adsorbent to phosphor for measurements, it was also necessary to investigate whether further variation is introduced by the presence of these adsorbing materials in the phosphor. To reduce this possible source of variation as much as possible exactly similar areas of paper were cut out for the examination of each spot; the papers were also orientated in the vials of phosphor in as identical a position as possible. Similar precautions were taken with the TLC work.

Instrumental error

Replicate counts of a particular sample of radioactive substance in phosphor showed that counts of more than 20,000 gave a coefficient of variation between 0.78 % (D-glucose-^{14}C(U)) and 0.45 % (morphine-2-T). It was therefore decided to use a minimum of 20,000 counts wherever possible.

Errors due to presence of paper or adsorbent in the phosphor

Known amounts of radioactive substances were delivered directly into vials of phosphor, and similar amounts delivered on to paper or TLC and then transferred to phosphor. Counts of each series were made at daily intervals and it was found that the means of the series containing paper or adsorbent were always less than those from the phosphor plus substance alone. However the radioactivities gradually increased and after a certain time interval reached a steady state and remained at this level for several days. For glucose this state was reached after 1 to 2 days, for tyrosine 2 days and for morphine 9 days. These results are probably partly due to slow elution of traces of substance from the paper or adsorbent. It was also noted that the coefficient of variation of the individual spots in each series was at a minimum when the steady state was reached. Future experiments were therefore designed so that a suitable time interval elapsed before counts were made.

Errors due to the measurement of small volumes of solutions

In published methods of quantitative chromatography volumes as small as 5, 2 or even 1 μl are quoted. These small volumes are measured by various instruments, the most popular being the Agla syringe, the Hamilton syringe or the Drummond Microcap micropipettes. Obviously a certain amount of experience is necessary to get maximum accuracy; accordingly we asked experienced workers to measure out 10 to 16 replicate volumes of solution with their favourite syringe (a) direct into phosphor and (b) on to paper or thin layer chromatograms. Exactly similar areas of paper or adsorbent containing each spot from (b) were transferred to phosphor and after a suitable time interval the radioactivities were determined. The results are shown in Table I.

Sources of error in measuring small volumes

The results in Table I, translated into terms of individual measurement, indicate errors ranging from $\pm$ 8 to $\pm$ 25 %, even with experienced workers using conventional syringes or pipettes. Since the errors are similar whether the volumes were measured direct into phosphor or via paper or adsorbent, the presence of the latter cannot be the source of variation. We have already shown that errors from the radioactive counting would not be more than $\pm$ 1.5 % so that the major source of error must be

TABLE I

ERRORS DUE TO MEASUREMENT OF SMALL VOLUMES OF SOLUTION BY EXPERIENCED WORKERS

Errors expressed as coefficient of variation (S.D. of individual results calculated as a percentage of the mean).

Worker	*Solute*	*Volume of solution measured* (μl)	*Coefficient of variation*	
			Via adsorbent (%)	*Direct into phosphor* (%)
A	Morphine-2-T	10 (Agla)	7.3 (Paper)	7.8
B	Morphine-2-T	10 (Agla)	10.1 (Paper)	5.8
C	Morphine-2-T	2 (Micropipette)	3.3 (Paper)	6.6
C	D-glucose-^{14}C(U)	2 (Micropipette)		4.7
		5 (Micropipette)		2.5
D	D-glucose-^{14}C(U)	1 (Hamilton)	8.2 (Paper)	11.1
	D-glucose-^{14}C(U)		7.8 (Paper)	10.4
	D-glucose-^{14}C(U)	1 (Hamilton)	8.8 (TLC)	
	D-glucose-^{14}C(U)		9.8 (TLC)	
B	D-glucose-^{14}C(U)	5 (Agla)		5.5
E	D-glucose-^{14}C(U)	5 (Agla)	3.2 (Paper)	3.4
	D-glucose-^{14}C(U)		6.0 (TLC)	
F	L-tyrosine-^{14}C(U)	5 (Agla)	9.1 (Paper)	4.5
	L-tyrosine-^{14}C(U)	10 (Agla)	11.9 (Paper)	10.9
B	L-tyrosine-^{14}C(U)	5 (Agla)		7.9
G	L-tyrosine-^{14}C(U)	5 (Agla)		4.7

variation in the volumes actually being delivered. A careful examination of the process of delivering the required volume indicated two sources of variation.

Creep back. The delivery of a number of drops from the needle of an Agla syringe by free fall was observed with a lens and it was noted that from time to time an accumulating drop suddenly slipped up slightly from the point and when the drop finally fell on to the paper a definite proportion remained on the stem. This "creep back" effect was cumulative and sometimes a sizeable volume remained on the stem for some time; then quite unpredictably it would disappear with a succeeding drop. The creep back effect varied with the solvent used and was particularly noticeable with methanol; occasionally after the delivery of many drops of a methanolic solution of morphine, crystals of the latter substance were seen to have formed as a tide mark on the stem of the needle. At times an exceptionally high tide of creep back would reach the crystals, redissolve them, and wash them into a succeeding drop.

Accordingly some of the needles used in the experiments recorded in Table I were washed after each series of radioactive solutions had been delivered. In several cases the "remainder" on the needle represented a significant proportion of the total radioactive substance measured, *e.g.* for morphine-2-T (Worker B) after delivering 16 volumes the remainder represented 9.6 % of the total delivered. Worker E, on the other hand, avoided creep back by using a hooked needle previously dipped in silicone; there was no detectable remainder after his series of deliveries.

Capillation. A second source of error arises from the fact that the measured drop does not always fall freely from the end of the needle as its weight may be insufficient to overcome surface tension effects. To assist this most workers touch the drop on to the surface of the paper or adsorbent or against the glass vial. We found that this process withdrew fluid from the lumen of the needle by capilliarity and sometimes it

TABLE II

ERRORS DUE TO MEASUREMENT OF SMALL VOLUMES OF SOLUTION USING THE MACHINE REFERRED TO IN THE TEXT

Worker	*Solute*	*Volume of solution measured* (μl)	*Coefficient of variation*	
			Via adsorbent (%)	*Direct into phosphor* (%)
B	Morphine-2-T	2	1.8	2.4
B	Morphine-2-T	5	2.5	2.2
B	Morphine-2-T	9.6	2.1	1.6
B	D-Glucose-^{14}C(U)	5	2.7	2.0
B	L-Tyrosine-^{14}C(U)	5	1.5	1.5

required the delivery of 0.6 to 0.8 μl of solution from the barrel of the syringe into the needle before the succeeding drop made its appearance. The amount withdrawn varied according to the bore of the needle, time of contact and state of absorbency of the absorbent material, which decreases as more liquid is added to it.

We attempted to overcome the two sources of error by (a) siliconing the needles, (b) using different bore thicknesses, and (c) filing the tapered ends till they were at right angles to the long axis, but none of these methods were entirely successful. Success was finally obtained with a machine which automatically delivered small volumes by rapid ejection or throwing. By this means creep back was prevented and since even quite small drops could be forced on to the paper or adsorbent without touching the surface, capillation was also prevented. This twofold advantage was demonstrated by producing a series of drops from the machine by forcible ejection in the normal way; the coefficient of variation was found to be $\pm$ 2.5 %. A second series was produced from the machine in identical circumstances except that the needle was brought sufficiently near to the paper for the drops to be drawn off by capillation, that is, to fall rather than be thrown. In these conditions the coefficient of variation rose to $\pm$ 7 %. Creep back had also occurred as the remainder left on the needle after this series represented 3.27 % of the total quantity delivered. When the normal throwing procedure was used the remainder was only 0.32 %.

In Table II some results obtained with the new machine are shown and it is obvious that the errors are considerably less than those for hand delivered drops (Table I). We hope to publish details of the machine shortly[13].

Towards the end of this work a machine* was marketed for the quantitative delivery of streaks of solution from a Hamilton syringe for band chromatography. Using the machine we collected ten volumes (30 μl each) of radioactive morphine solution direct into phosphor and found the coefficient of variation was $\pm$ 4.4 %. Another device** delivering small volumes by ejection was tested; analysis of ten volumes (25 μl each) gave a coefficient of variation of 9.74 %.

DISCUSSION

This work has brought to light a major source of error in quantitative chromatography which has probably been hitherto largely unsuspected. The results recorded

* Camag Chromatocharger, Griffin & George Ltd., London.
** Repeating Dispenser, Shandon Scientific Company Ltd., London.

in Table I were based on delivery of normal volumes of solutions by very experienced workers using their own syringes or pipettes yet the errors of individual results were frequently of the order of 20 % or more of the mean. This is great deal more than would be expected, say, of the Agla syringe whose makers claim an error of $\pm$ 0.05 μl; on a delivered volume of 10 μl this should represent only 0.5 %, yet workers A, B and F, using 10 μl volumes from an Agla, obtained errors up to 20 % and more. We have shown that these errors are mainly due to creep back and capillation, errors which obviously only arise when a series of volumes are delivered in succession. Where a single delivery is made with a micropipette (Worker C) the errors are significantly less. However for many operations replicate volumes, such as are delivered by an Agla or Hamilton syringe, are required. One of us (S.J.R.)[13], therefore, has devised a machine to overcome these defects and its use not only reduces errors markedly (Table II) but avoids the fatigue normally involved in delivering a succession of volumes.

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