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# RADIOACTIVITY MONITOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

The coupling of a homogeneous radioactivity monitor to a liquid chromatograph involves compromises between the sensitivity of the monitor and the resolution and speed of analysis of the chromatograph. The theoretical relationships between these parameters are considered and expressions derived which make it possible to calculate suitable monitor operating conditions for most types of high-performance liquid chromatography.

## INTRODUCTION

The detection and measurement of radioactivity in the eluent from a liquid chromatographic (LC) column presents few technical problems. In general, all that is required is that successive fractions be collected and each analysed by liquid scintillation counting. However, since the advent of high-performance liquid chromatography (HPLC), this procedure has become time-consuming because large numbers of small fractions must be collected and assayed in order to maintain chromatographic resolution. Radioactivity determinations can therefore extend the duration of an HPLC analysis from a matter of minutes to several hours, and as a consequence much of the practicality of HPLC is lost. For this reason continuous-flow monitoring is desirable if HPLC is to be effectively utilized in the analysis of radioactive compounds.

The continuous-flow monitoring of $\beta$ radiation in LC eluents has usually involved the use of a scintillation technique, and, depending upon the method of presentation of the eluent to the scintillator, can be classified as either a heterogeneous or a homogeneous system[1]. In the latter instance the column eluent is mixed with a liquid scintillation cocktail before passing through a flow cell positioned between the photomultiplier tubes of a liquid scintillation counter. In heterogeneous systems the eluent moves directly to the flow cell which is packed with a finely divided solid scintillator such as anthracene, PPO, scintillator plastic, or Ce-activated Li glass.

Heterogeneous counting systems are free of chemical quenching effects and the sample can be easily recovered. However, they exhibit relatively low counting efficiencies for the low-energy $\beta$ emitters. For example, with tritium the figure is

generally well below 1 %. The versatility may be further affected by sample adsorption and solubility of the scintillator in the mobile phase[1]. Heterogeneous detectors are consequently best suited to LC systems when the levels of radioactivity in the column eluent are high and the main requirement is purification.

Homogeneous, continuous-flow detectors are best used in conjunction with analytical chromatographic procedures, such as HPLC, where recovery of the sample is unimportant relative to other considerations such as sensitivity and versatility. The technique is also applicable to preparative LC when a suitable eluent splitter is used.

Although a review of heterogeneous counting systems has been published[1] the limited information available on homogeneous detectors[2,3] is unrelated to basic chromatographic parameters and therefore of little direct value in the application of the technique to HPLC. This report attempts to remedy the situation by considering theoretical relationships between chromatographic processes and the measurement of radioactivity. On the basis of this theory a generalised procedure is described for the design of homogeneous radioactivity monitors suitable for use with a wide range of LC techniques including HPLC.

## MATERIALS AND METHODS

A basic homogeneous scintillation detector system for HPLC is illustrated in Fig. 1. The mobile phase was delivered from the solvent reservoir to the column inlet by a pulse-free high pressure pump. Microlitre-size samples were introduced into the column inlet via an injection head. Solutes emerging from the column outlet were passed through the UV monitor flow cell before being mixed, at the low dead volume "T", with scintillation cocktail delivered from a micrometering pump via a pulse-dampening network comprised of a micrometering valve and two pressure gauges.

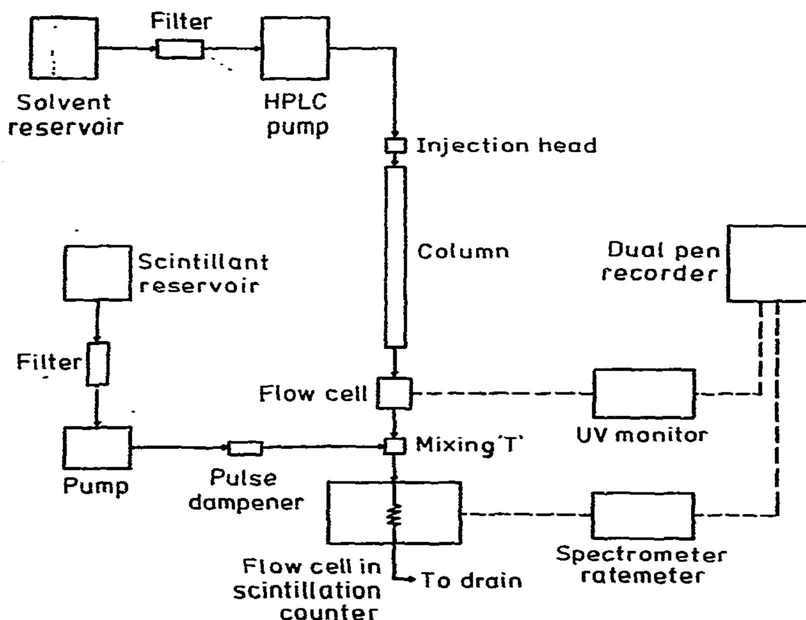


Fig. 1. Schematic of an HPLC fitted with a continuous-flow radioactivity detector.

The scintillant–eluent mixture was then passed through a spiral glass flow cell in a Coruflow manual scintillation counter connected to a spectrometer–ratemeter (ICN Pharmaceuticals, Hersham, Great Britain). The counting rate was displayed on a chart recorder along with the output from the UV monitor.

## RESULTS AND DISCUSSION

### *Theoretical considerations*

Because of the unusual characteristics of homogeneous radioactivity monitors it is necessary to compromise detector sensitivity with both chromatographic resolution and speed of analysis. The nature of the compromise is central to the design of an efficient detector system and will consequently be discussed in some detail.

The output of any continuous flow detector system can be considered to be the net result of a large number of discrete independent measurements of samples equal in size to the volume of the flow cell, $V_{cell}$, averaged over a period of time equal to the transit time of the solute through the cell, $t_{tr}$. Thus $V_{cell}$ and $t_{tr}$ determine the minimum volume and time period over which a change in detector response can be recorded. It is important that the $V_{cell}$ be as small as possible in order to reduce band-spreading originating from the detector. However, this presents a particular problem with radioactivity detectors because they respond to the total amount of activity in the flow cell and thus any reduction in $V_{cell}$ will be accompanied by a parallel reduction in sensitivity. In contrast, the volume of the flow cell of a UV monitor can be reduced without affecting the sensitivity, provided that the optical pathlength remains unchanged, since the response is derived from concentration per unit path length. In the case of a radioactivity monitor it is evident that a compromise between chromatographic resolution and detector sensitivity must be reached, the exact nature of which depends upon the requirements of the analysis. Where the maximum resolving power of HPLC is required, for example in the analysis of complex mixtures, band-spreading arising from the detector cannot be tolerated and this can only be avoided by sacrifices in the sensitivity of the radioactivity monitor. Alternatively, if only low resolution is required, such as in the analysis of simple reaction mixtures, the flow cell volume can be increased in order to enhance overall sensitivity and improve quantification.

The compromise between sensitivity and resolution is most readily quantified by the ratio between the cell volume and the effective width of the peak, $w^s$. The value of $w^s$ is related to the volume of the true chromatographic peak width, $w$, by $w/X$, where $X$ is the proportion of eluent in the scintillant–eluent mixture. In practice, $w^s/V_{cell}$ ratios as low as 10 can be tolerated without a significant loss of resolution. The effective peak width (ml) of a component eluting with a capacity factor, $k'$, and efficiency, $N$, from a column with void volume ($V_0$) can be calculated from eqn. 1:

$$w^s = \frac{4\,V_0\,(k'+1)}{\sqrt{N}\cdot X} \tag{1}$$

Thus, for most practical purposes $V_{cell}$ (ml) can be set as

$$V_{cell} = \frac{0.4\cdot V_0\,(k'_R+1)}{\sqrt{N}\cdot X} \tag{2}$$

where $k'_R$ is the capacity factor of the least retained component of interest. Components eluting with $k'$ values greater than $k'_K$ will be detected with lower sensitivity and higher resolution.

The second compromise which has to be reached in the design of a radioactivity monitor is that between sensitivity and speed of analysis. The precision of any estimate of radioactivity depends statistically upon the number of events contributing to that measurement, hence the longer the measurement period the more accurate the result. However, in the case of continuous-flow counting, the measuring period is determined by transit time, and this in turn is linked to the speed of analysis via the flow-rate. Thus the detector sensitivity and column flow-rate will always be strongly interdependent. The nature of this relationship is of fundamental importance to the design of a detector for HPLC where flow-rates are high and, as a consequence, transit times are barely adequate.

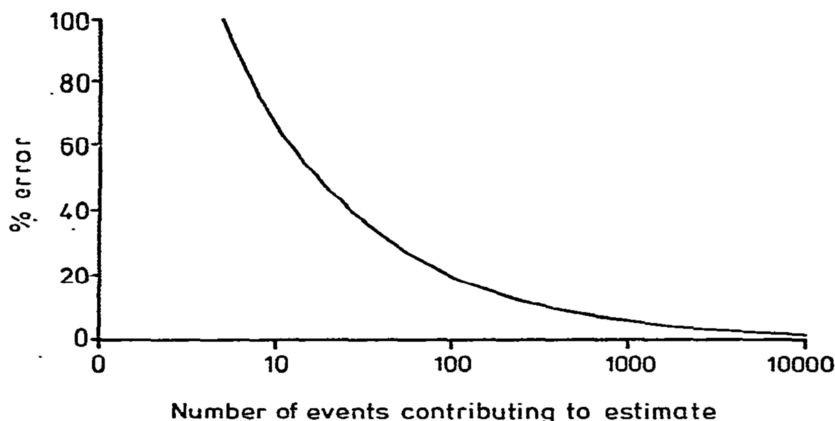


Fig. 2. Relationship between the percentage error of an estimate of counting rate and the total number of counts contributing to that estimate. Taken from the 95% confidence limits of $\lambda$ (ref. 4).

Fig. 2 is plotted from a Poisson distribution[4] and indicates the approximate percentage error on a measurement made up of a given number of discrete, independent radioactive disintegrations. It can be seen that at least 100 radioactive disintegrations must be recorded within the transit time if a statistically valid estimate of the count rate is to be made. Furthermore, it is apparent that after some 100 to 200 counts have been recorded, any further increase in the accuracy of the rate measurement demands greatly increased counting times. Therefore, in practical terms, most of the necessary information is acquired by the accumulation of 100–200 counts, and a disproportionate amount of time must be invested if an accuracy of better than 10–20% is required. If fewer than 100 counts are accumulated, the percentage error will be very much larger. The factors controlling the quantitative precision of estimates of total radioactivity by on-flow detectors for LC have been thoroughly discussed by Sieswerda *et al.*[5]. However, when the information sought is of a qualitative nature the detection of a peak containing less than 100 counts can still be of some qualitative value despite the gross errors in quantification. Under such circumstances it is of course important that the background counting rate be minimised.

The foregoing considerations make it desirable to define two types of detector sensitivity:

(a) absolute sensitivity, *i.e.*, the amount of activity that must be present within the cell to ensure a 95% probability of maintaining a detector response of better than twice the background over a period equal to the transit time, and

(b) absolute quantitative sensitivity, *i.e.*, the amount of activity that must be present within the cell in order to obtain an average of 100 counts per transit period. The absolute sensitivity, $AS$ (dpm), of a detector can be derived as

$$AS = \frac{6 \cdot \lambda^s}{t_{tr} \cdot E} \times 10^3 \quad (3)$$

where $t_{tr}$ is the average transit time (sec), $E$ is the percentage counting efficiency, and $\lambda^s$ characterises the Poisson distribution associated with the average number of sample counts per transit period.

The parameter $\lambda^s$ is related to the background activity in the following manner. If the instrument background is $Bg$ cpm then during the transit time, $t_{tr}$ sec, an average of $Bg \cdot t_{tr}/60$ events will be measured, any variation being described by the Poisson distribution $\lambda^{Bg} = Bg \cdot t_{tr}/60$. If $\lambda_r^{Bg}$ is the upper 95% confidence limit of this distribution, then in order to ensure a 2:1 signal-to-noise ratio for the period the sample is in the detector, counts attributable to the sample must be at least equal to the value of $\lambda_r^{Bg}$. If the Poisson distribution given by $\lambda^s$ describes the variation in the number of sample counts accumulated in time, $t_{tr}$, and $\lambda_l^s$ is its lower 95% confidence limit, then $\lambda_l^s$ must be equal to or greater than $\lambda_r^{Bg}$. Thus, if the values of the instrument background and transit time are known, it is possible to compute $\lambda^s$ from Poisson tables[4] and given the percentage counting efficiency, obtain the absolute detector sensitivity.

The absolute quantitative sensitivity, $AS_q$ (dpm), is simpler to derive:

$$AS_q = \frac{6}{t_{tr} \cdot E} \times 10^5 \quad (4)$$

This relationship is approximate as it does not account for background activity. However, as few background counts are accumulated in the short transit times common to most HPLC applications, it is usually possible to disregard contributions from this source.

Because at any one time only a portion of the sample peak is within the sensitive volume of the flow cell, the actual amount of radioactivity which must be injected to evoke a detector response will always be greater than that indicated by the absolute sensitivity figure. It is therefore useful to define two practical estimates of system sensitivity:

(a) relative sensitivity, *i.e.*, the minimum amount of sample which must be injected to give at least a 95% chance of doubling the maximum noise level likely to be encountered during any one transit period, and

(b) relative quantitative sensitivity, *i.e.*, the minimum amount of sample which must be injected to obtain an average of 100 counts per transit period.

In the first instance, the relative sensitivity is related to the absolute sensitivity by the ratio $w^s/V_{cell}$. However, this does not take into account the peak shape which often approaches the ideal Gaussian form. This means that the sample concentration

at peak maximum is approximately twice the average sample concentration and hence only half the amount of sample indicated by the expression $(w^s/V_{cell})\cdot AS$ need be injected into the chromatograph in order for the radioactivity monitor to detect the peak maximum. However, if the presence of a compound is to be convincingly demonstrated, it is usually necessary to obtain information on peak shape as well as to determine its maximum point. Consequently, $(w^s/V_{cell})\cdot AS$ more closely approximates the relative sensitivity than $(w^s/V_{cell})\cdot AS/2$.

A similar argument can be applied to the relative quantitative sensitivity where most of the peak has to be above the minimum quantitatively meaningful count rate if an accurate peak area estimate is to be obtained. Thus $(w^s/V_{cell})\cdot AS_q$ is a better measure of the relative quantitative sensitivity than $(w^s/V_{cell})\cdot AS_q/2$. Substituting for $AS$ and $AS_q$ in eqns. 3 and 4 gives the relative sensitivity, $RS$ (dpm):

$$RS = \frac{6\cdot\lambda^s\cdot w^s}{t_{tr}\cdot E\cdot V_{cell}} \times 10^3 \quad (5)$$

and the relative quantitative sensitivity, $RS_q$ (dpm):

$$RS_q = \frac{6\cdot w^s}{t_{tr}\cdot E\cdot V_{cell}} \times 10^5 \quad (6)$$

These equations become more useful if applied to the general case where allowance has to be made for the variation of $w^s$ with $k'$. Thus if it is assumed that $N$ is effectively independent of $k'$, as is commonly the case with modern HPLC columns, substitution of eqn. 1 into eqns. 5 and 6 gives

$$RS = \frac{2.4\cdot V_0\,(k'+1)\,\lambda^s}{\sqrt{N}\cdot X\cdot t_{tr}\cdot E\cdot V_{cell}} \times 10^4 \quad (7)$$

$$RS_q = \frac{2.4\cdot V_0\,(k'+1)}{\sqrt{N}\cdot X\cdot t_{tr}\cdot E\cdot V_{cell}} \times 10^6 \quad (8)$$

From eqn. 5 it can be seen that for a given set of operating conditions RS $\propto \lambda^s/t_{tr}$. Since $\lambda^s$ is derived from $t_{tr}$, the influence of transit time on the relative sensitivity is determined by the nature of the expression $\lambda^s/t_{tr}$, which is such that as $t_{tr}$ becomes large $\lambda^s/t_{tr}$ approaches a constant. The background count rate determines the transit time at which this occurs. This is illustrated in Figs. 3 and 4 for the optimum case in which $w^s/V_{cell} = 10$. Fig. 3 is calculated using the typical tritium counting parameters of 25 cpm background and 25% efficiency. Provided the flow-rate is chosen to give a transit time of 15–20 sec a relative sensitivity of 3,000 dpm will be achieved. Any further increase in transit time will do little to improve the sensitivity. If typical $^{14}C$ counting parameters ($Bg$ = 12 cpm, $E$ = 80%) are used the optimum transit time is approximately 30 sec and the relative sample sensitivity approximately 500 dpm (Fig. 4). Thus the optimum transit time is heavily dependent upon the background of the instrumentation.

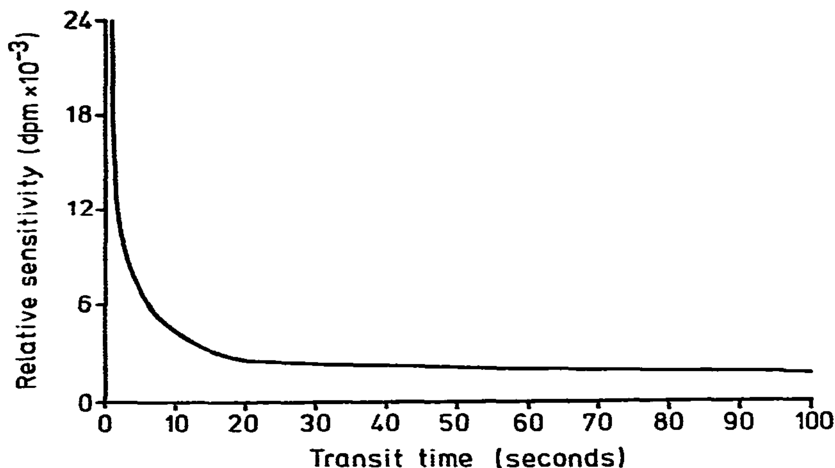


Fig. 3. The influence of transit time on the relative sensitivity of a radioactivity detector when $w^s/V_{cell} = 10$, $E = 25\%$ and $Bg = 25$ cpm.

*Design procedure*

Because of the number of interrelated factors involved, it is important to consider the design variables in the correct sequence. Such a sequence is outlined below and by similar calculations it should be possible to derive an accurate estimate of the relative sensitivity and time of analysis of any given system without recourse to extensive experimentation.

*Chromatographic system*

Although many commonly used solvents are quenching agents a homogeneous continuous-flow radioactivity monitor will perform satisfactorily with most HPLC systems. When the degree of quenching and subsequent loss of sensitivity is unac-

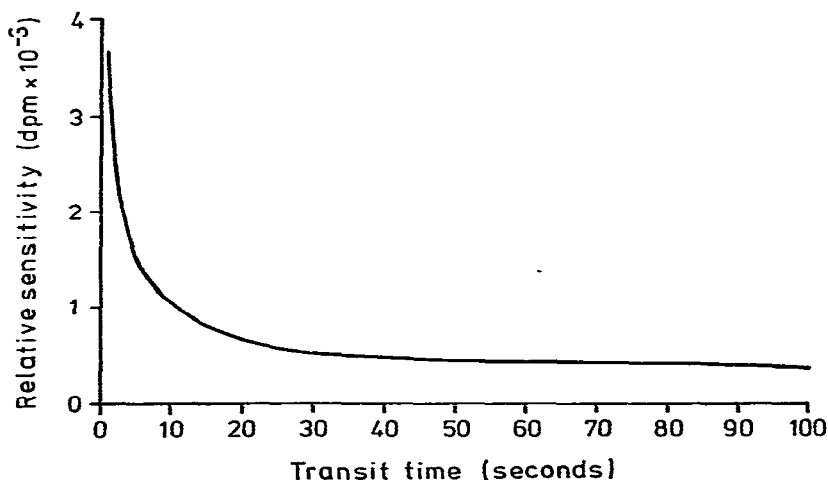


Fig. 4. The influence of transit time on the relative sensitivity of a radioactivity detector when $w^s/V_{cell} = 10$, $E = 80\%$ and $Bg = 12$ cpm.

ceptable it may be possible to replace the offending mobile phase component with a more suitable solvent. If this approach fails most analyses can be carried out with an alternative HPLC system using a mobile phase compatible with high detector sensitivity.

*Scintillation cocktail*

Chemical quenching is nearly always evident, except in the case of the analysis of non-polar compounds using benzene, toluene, hexane and heptane mobile phases, and is best combated by the use of dioxane–naphthalene scintillation mixtures. The optimum cocktail composition may be estimated by efficiency determinations on a range of scintillant–eluent mixtures by conventional liquid scintillation counting, using either internal standardisation or channels-ratio methods. Varying dioxane–eluent ratios (*e.g.*, 1:5, 1:2, 1:1, 2:1, 5:1 and 10:1) containing naphthalene (100 g/l), PPO (7 g/l) and POPOP (0.3 g/l) should be tested. Counting efficiency improves as the proportion of mobile phase to dioxane decreases, although excessively high scintillant–eluent ratios result in inconvenient flow-rates and the consumption of excessive volumes of scintillant. In general, the optimum ratio varies from approximately 1:5 in the case of solvents such as ethyl acetate to 10:1 for aqueous mobile phases. A list of scintillant–eluent mixtures and the corresponding counting efficiencies for a range of typical HPLC solvents is given in Table I. These values can be shown to depend upon solvent purity and the presence of modifiers. The data in Table I were obtained using crude "drum" dioxane and bulk naphthalene, and therefore could be improved through the use of scintillation grade chemicals. The efficiencies for aqueous solvents are low and could be increased by employing detergent-based emulsifying agents such as Triton X-100. An additional advantage of this type of system would be a reduced susceptibility to quenching by salts in the mobile phase.

TABLE I

TYPICAL SCINTILLANT–ELUENT RATIOS AND COUNTING EFFICIENCIES OBTAINED FOR VARIOUS CLASSES OF HPLC SOLVENTS

Average background 20–30 cpm for $^{3}H$ and 10–15 cpm for $^{14}C$.

| *Solvent class* | *Scintillant–eluent ratio* | *Counting efficiency (%)* | |
|---|---|---|---|
| | | $^{3}H$ | $^{14}C$ |
| Hydrocarbons | 1:2 | 35 | 80 |
| Esters | 1:2 | 25–30 | 75–80 |
| Chlorinated hydrocarbons | 1:1 | 12–18 | 60–70 |
| Aqueous | 10:1 | 8–15 | 60–70 |

*Optimum transit time*

The values for efficiency and background obtained by conventional liquid scintillation counting of scintillant–eluent mixtures in vials will closely approximate those associated with a flow cell. From the background value, it is possible to calculate $\lambda^{s}$ for a range of transit times using Poisson distribution tables[4], and to substitute the value of $\lambda^{s}$ and that of the efficiency $E$ in eqn. 5. Except in those instances where resolution has to be sacrificed for sensitivity, the $w^{s}/V_{cell}$ ratio should be 10. The variation of relative sensitivity with transit time can then be plotted as in Figs. 3 and 4. From

such a plot the $t_{tr}$ value providing the best compromise between sensitivity and speed of analysis can be selected. At this early stage of design it is possible to decide whether or not the sensitivity is adequate. If not, several possibilities for improvement exist. Although in theory, the ratio $w^s/V_{cell}$ could be reduced to a value less than 10, in practice the loss in resolution will be noticeable and in most cases unacceptable. However, considerable improvement in sensitivity may be obtained by modifications of the mobile phase or scintillation cocktail, or of the scintillant–eluent ratio. In cases where speed of analysis is not a critical factor and the background can be further reduced, sensitivity can be considerably improved by increasing the transit time.

The ratemeter time constant should ideally be matched with the transit time. However, because of the limited choice of time constant settings on most ratemeters and the non-linear integration characteristics of the electronic time constant, it is acceptable to select a time constant value higher than the transit time, *e.g.*, a 6-sec transit time, and a 10-sec time constant.

*Flow cell*

In order of importance the critical parameters governing flow cell design are cell volume, cell geometry and the materials of construction.

*Flow cell volume.* The cell volume can be calculated from a chromatogram (obtained with a UV or RI monitor) that is typical of the separations being undertaken. The width of the narrowest peak of interest, $w$, can be calculated in millilitres and by definition the cell volume, $V_{cell}$, will have to be $1/10 \cdot X$ of this volume, where $X$ is the proportion of the eluent in the scintillant–eluent mixture. Only in instances where it is expedient to sacrifice resolution for sensitivity will it be possible to use a larger flow cell than that calculated on this basis.

Having thus determined the value of $V_{cell}$, and using the values for $X$ and $t_{tr}$ established in the two preceding sections, the column flow-rate, $f$ (ml/min), must be

$$f = \frac{V_{cell} \cdot X}{60 \cdot t_{tr}} \tag{9}$$

Ideally the calculated column flow-rate should be similar to that typically used for the chromatograph. However, if the flow-rates differ considerably, the test substances should be re-run using the presumably lower calculated flow-rate to ascertain if the value of the minimum peak width has been altered. With modern HPLC columns peak width is not greatly influenced by small changes in flow-rate provided this is not unduly slow. However, in most classical LC systems relatively small changes in flow-rate can significantly affect peak width. In these circumstances, the only means of obtaining the optimum value of both $V_{cell}$ and $f$ is to solve by graphical means the simultaneous equations $V_{cell} = z_1 \cdot f$ and $V_{cell} = z_2 \cdot f$, where $z_1 = t_{tr} \cdot 60/X$, which has already been obtained in eqn. 9 and $z_2$ is the complex constant of the relationship of the required cell volume to flow-rate, and is empirically obtained by plotting $1/10\ w^s$ against $f$.

*Flow cell geometry.* The most important aspect of cell geometry is the relationship between cell bore and cell volume. It is essential that this ratio be as small as possible so as to avoid peak trailing. However, if the cell internal diameter (I.D.$_{cell}$) is extremely small, large pressure differentials result. Furthermore, the required cell volume can be obtained only by increasing the length of the tubing with consequent

difficulties in accommodating the flow cell within the counting chamber. In practice, the dimensions of flow cells fall within the limit $V_{cell}/(I.D._{cell})^2 > 2$ but $<3$ where $V_{cell}$ is the cell volume (ml) and $I.D._{cell}$ is the internal diameter of the cell (mm). It will be found from the above relationship that 1.5–2.5 m of tubing is required to construct a cell of suitable geometry.

The physical arrangement of the cell within the counting chamber is of secondary importance. Maximum light transmission is obtained from a flat spiral of the same diameter as the photomultiplier tubes which is sandwiched between the opposed faces of the two tubes. In practice a random arrangement of the flow cell tubing is found to provide only slightly inferior results. Because of the length of the tubing involved, a multi-layered close-wound spiral is the most convenient to construct.

*Materials of construction.* Any relatively transparent, chemically inert, narrow-bore tubing made of PTFE, polypropylene or glass may be used. Because of the high surface area to volume ratio of the flow cell, the transparency of the wall material is more critical than in the case of a scintillation vial. Very high background counting rates can result from the large amount of wall material present within the counting chamber, especially when thick-walled glass tubing is being used. Whilst glass is generally preferable to plastic for flow cell construction, relative transparency alone should not be the sole criterion for distinguishing a suitable cell material. The efficiency gained from highly transparent materials will be offset if the background is also increased. For this reason the background associated with a range of different types of tubing should be investigated before the cell is finally constructed. This determination can be carried out on 15-cm lengths of each sample of tubing provided that these have similar I.D. The tubing is cut into sections small enough to fit into a standard 20-ml scintillation vial. The scintillant–eluent formulation as determined above (see Scintillation cocktail) is then added to each vial and the background, its decay rate and energy distribution examined by conventional liquid scintillation counting.

Most inert plastic tubings after exposure to ambient light show relatively strong phosphorence, though this typically has a rapid decay rate and is, therefore, of little consequence provided the cell is maintained in darkness. Phosphorescence in glass tends to be longer-lived, so, if present, can cause some inconvenience. It is common for both plastic and glass to exhibit chemiluminescence-like effects and for this reason certain batches of PTFE tubing must be rejected. Some glasses appear to interact with the scintillant and even compounds eluting from the column to produce very high counting rates. Typically the energy distribution associated with this phenomenon is relatively narrow but is inconveniently centred near the tritium spectrum. Fortunately, it can be virtually eliminated by pretreatment of the glass with 10% orthophosphoric acid. It is a useful precautionary measure to treat all glass flow cells in this manner prior to use. A certain amount of the background in some glass cells can be attributed to radioactive potassium, but as glass tubing with a low potassium content can be obtained, the problem is readily overcome.

*Other points*

When coupling the radioactivity monitor to the HPLC, care should be taken to reduce peak tailing by the use of adequately narrow-bore tubing and fittings and by minimising the length of the interconnecting lines. With a little care it is possible

to maintain the resolution of a peak as narrow as 300 $\mu$l. One less obvious potential source of band-spreading occurs when a reciprocating piston pump is used to deliver the scintillant. If the displacement volume of the pump is similar to the chromatographic peak width then mixing at the "T" becomes significant because of pulsation and must be eliminated by means of a pulse dampener in the scintillant delivery line (Fig. 1).

Eluents from preparative liquid chromatographs may display unusual behaviour when coupled to a homogeneous continuous flow radioactivity monitor. Sporadic, high backgrounds can arise through mixing of the scintillant and column eluent, as well as from the high velocities of scintillant–eluent mixture in the narrow-bore delivery lines. Cooling the solution to 0° for about 30 sec is often sufficient to com-

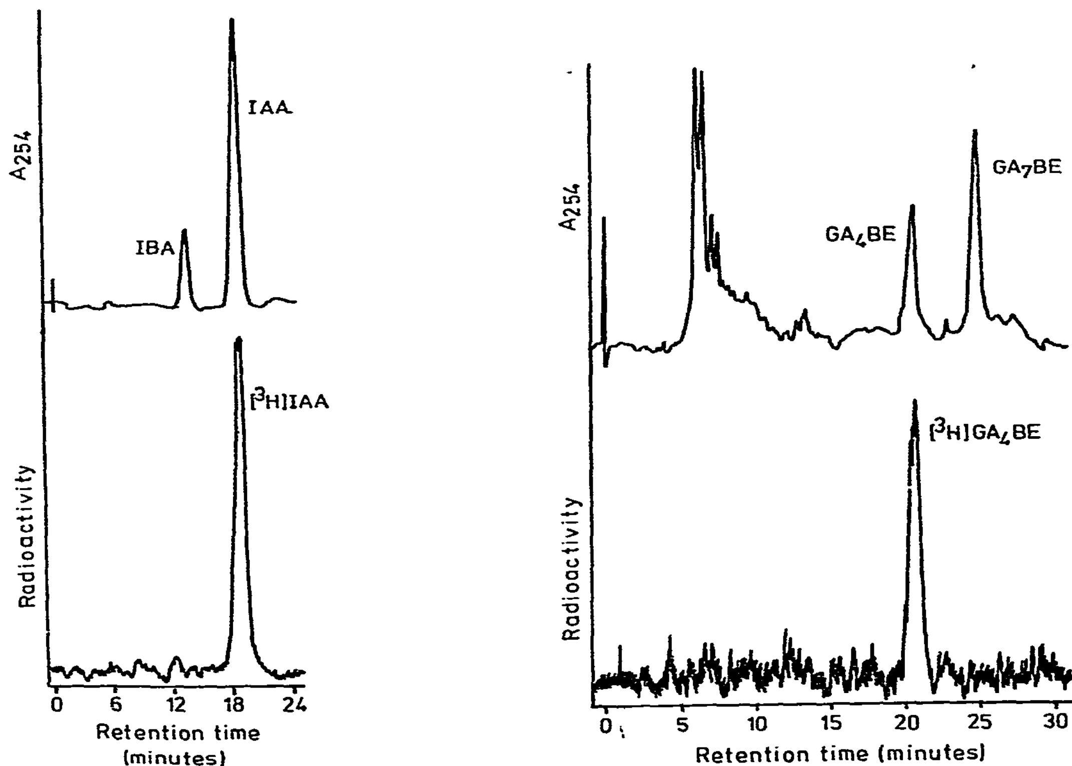


Fig. 5. Preparative HPLC of [$^3$H]indole acetic acid and indole butyric acid. Column, 10 × 450 mm Partisil 10; mobile phase, 40% ethyl acetate in hexane; stationary phase, 43%, 0.5 *M* formic acid; $V_0$, 27.0 ml; flow-rate: 5.0 ml/min; scintillant, 200 g naphthalene, 14 g PPO, 0.6 g POPOP per litre of dioxane; *X*, 0.71; *E* for $^3$H, 25%; *Bg*, 28 cpm; $V_{cell}$, 1.1 ml; $t_{tr}$, 9 sec; ratemeter time constant, 20 sec; ratemeter range, 600 cpm. For [$^3$H] IAA: *k'*, 2.4; *N*, 1400; UV peak width, 117 sec; radioactivity peak width, 126 sec.

Fig. 6. Analytical HPLC of [$^3$H]gibberellin $A_4$ benzyl ester and gibberellin $A_7$ benzyl ester. Column, 4.6 × 500 mm Partisil 10; mobile phase, 1% dimethyl sulphoxide in hexane–dichloromethane (1:1) saturated with water; $V_o$, 6.0 ml; flow-rate, 1 ml/min; scintillant, 200 g naphthalene, 14 g PPO, 0.6 g POPOP per litre of dioxane. *X*, 0.5; *E* for $^3$H, 20%; *Bg*, 35 cpm; $V_{cell}$, 200 $\mu$l; $t_{tr}$, 9 sec; ratemeter time constant, 10 sec; ratemeter range, 600 cpm. For [$^3$H] $GA_4BE$: *k'*, 2.3; *N*, 4600; UV peak width, 72 sec; radioactivity peak width, 78 sec.

pletely suppress these effects. This can be achieved by passing the scintillant–eluent mixture through a suitable length of narrow-bore stainless-steel tubing immersed in a cooling bath before it enters the flow cell.

*Use of the radioactivity monitor*

The application of the foregoing design principles to two differing HPLC systems is illustrated in Figs. 5 and 6. Fig. 5 illustrates the separation of [$^3$H]-indole acetic acid (IAA) from indole butyric acid (IBA) by a preparative HPLC system. The chromatographic parameters of this analysis along with those derived for the radioactivity monitor are presented in the legend of Fig. 5 and it can be seen that the condition $w^s/V_{cell} > 10$ will be met for solutes with $k' > 1.7$. Thus, [$^3$H]-IAA eluting with a $k'$ value of 2.4 has a peak width of 117 sec as measured on the UV monitor and 126 sec as measured on the radioactivity monitor, indicating that the extra-column bandspreading due to the radioactivity monitor amounts to only 14% of the total peak variance[6]. The relative sensitivity and relative quantitative sensitivity can be calculated from eqns. 7 and 8 as $6 \times 10^3$ and $33 \times 10^3$ dpm respectively. In Fig. 5 $36 \times 10^3$ dpm was injected into the chromatograph and, as can be seen from the trace, the estimate of $6 \times 10^3$ dpm for the relative sensitivity limit is acceptably precise.

The separation of an impure mixture of [$^3$H]-gibberellin $A_4$ benzyl ester ($GA_4BE$) and gibberellin $A_7$ benzyl ester ($GA_7BE$) by analytical HPLC is illustrated in Fig. 6. The parameters applicable to this typical HPLC situation are also listed in the figure legend. The cell volume was chosen to give $w^s/V_{cell} > 10$ for $k' > 1.8$. The radioactivity monitor resolution obtained for solutes with $k' < 1.8$ was considered unimportant because all but the simplest of separations require $k'$ values $>1.5$ in order to generate a sufficiently high effective plate count. [$^3$H]-$GA_4BE$ elutes with a $k'$ of 2.3 and thus has a peak width of 72 sec on the UV monitor and 78 sec on the radio activity monitor. This means that the radioactivity monitor is contributing 15% to the total peak variance. Because of the low counting efficiency associated with the dichloromethane mobile phase and the relatively high background of the flow cell the optimum transit time was only 6 sec. In spite of this low $t_{tr}$ value sensitivities are still adequate with $RS = 9.3 \times 10^3$ dpm and $RS_q = 58 \times 10^3$ dpm. The detector response to $28 \times 10^3$ dpm of [$^3$H]-$GA_4BE$ is in agreement with these estimates.

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