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MONITORING RADIOACTIVE COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ELUATES: FRACTION COLLECTION VERSUS ON-LINE DETECTION

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SUMMARY

This study compared two methods of monitoring radioisotopes in high-performance liquid chromatographic eluates (on-line radioactivity detector versus fraction collection and counting). Testing was accomplished by pumping solutions of tritiated water in acetonitrile—water mixtures through the detector or to the fraction collector. At most solvent compositions, the detector's counting efficiency and detection limits were poorer than those of the scintillation counter. However, the reproducibility of the detector data was superior at acetonitrile concentrations of less than 50%. This was attributed to the difficulty in collecting fractions of small equal volumes at the lower organic solvent concentrations in short time intervals. We conclude that on-line monitoring with homogeneous detection is the preferred method for detecting radiolabeled compounds in high-performance liquid chromatographic eluates.

INTRODUCTION

Biochemical studies frequently involve the use of radiolabeled compounds whose movements and transformations can be monitored in biological systems. The degree of information obtained from these experiments is in part dependent on the procedures and tools designed to analyze biological materials. The ability to isolate, characterize, and quantitate radioactive products is at the foundation of experiments utilizing radiolabeled tracer molecules.

In recent years, high-performance liquid chromatography (HPLC) has emerged as another technique for separating radiolabeled compounds. The detection of radioactive components in HPLC eluates though is complicated by the characteristic small sample sizes, narrow peak widths, and small peak volumes of the method. This necessitates radioactivity measurements in small volumes of eluate to resolve closely eluting peaks.

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Fraction collection followed by quantitation in a liquid scintillation counter has been one common approach used with β-emitters (^{3}H, ^{14}C, ^{35}S, ^{32}P). Usually, equal volume fractions are collected on a time basis and coordinated with the output of an in-line spectrophotometric, fluorometric or electrochemical detector. This is an expensive, time-consuming, and labor-intensive procedure though that requires aliquoting to scintillation vials, addition of scintillation fluid, capping of vials, and loading and unloading the scintillation counter. The data output is generally in an inconvenient form as a list of numbers which must be plotted to visualize the chromatogram. However, there is an advantage to collecting and counting since there is no time constraint on the measurement of the radioactivity in the fractions and the precision of counting can be improved by increasing the counting time. Although this detection technique is costly and inconvenient, it should give good results if small reproducible fraction volumes can be obtained.

Radiolabeled compounds in HPLC eluates can also be monitored in real-time with a flow-through on-line detector. This can greatly reduce operating expenses and labor. Two different types of flow cells (heterogeneous and homogeneous) have been used to detect β-emitters. Heterogeneous flow cells are packed with a solid scintillator, whereas in homogeneous systems, the HPLC eluate is mixed with a non-gelling scintillator fluid before entering the flow cell. The primary advantage of the heterogeneous approach is that the separated components of the sample can be recovered after detection. Nevertheless, heterogeneous detectors exhibit a variety of problems. These include: (1) low counting efficiencies for low-energy β-emitters such as tritium [1]; (2) interaction of the sample with the solid scintillator resulting in peak broadening or irreversible sorption on the scintillator [2]; (3) contamination of the sample by the solid scintillator [1]; and (4) high back-pressures that may exceed the limits of other in-line detectors [3]. However, heterogeneous flow cells in radioactivity flow detectors are recommended when the radioactivity in the sample is high and the main requirement is purification [2].

In contrast, the homogeneous flow cell system in a radioactivity flow detector is more sensitive and trouble-free than the heterogeneous type. Its main drawback is that the sample is irreversibly destroyed when the eluate is mixed with a scintillator fluid. However, this problem can be minimized with the use of a stream splitter to divert a percentage of the eluate to a fraction collector [1, 4]. At the present time, homogeneous detection is the most popular approach used for detection of β-emitters.

This study was designed to critically compare the methods of fraction collection followed by counting and on-line homogeneous detection. Counting efficiencies, precisions and detection limits have been determined using short counting and collection intervals (0.2 min) and varying solvent compositions (0—100% acetonitrile). A simple detector evaluation procedure is presented that involves pumping solutions of a labeled compound (tritiated water) directly through a detector without a column or injector.

EXPERIMENTAL

Static counting

The characteristics of the radioactivity monitor (FLO-ONE/DR, Radiomatic

Instruments and Chemical Co., Tampa, FL, U.S.A.) under non-flowing conditions were evaluated by using a syringe to fill the 0.5-ml flow cell with test mixtures and recording counts. The solutions were prepared by mixing scintillator (FLO-SCINT II, Radiomatic Instruments and Chemical Co.) with 20, 40, 60, 80 and 100% acetonitrile at a volume ratio of 3:1 (scintillator:solvent). The static counts in 0.2-min intervals were recorded over a 5-min period (25 measurements). A mixture of scintillator plus water (0% acetonitrile) proved to be too viscous to fill the cell using a syringe. The same solutions containing 50 000 dpm/ml tritiated water (Sigma, St. Louis, MO, U.S.A.) were examined in an identical manner. These static counting experiments were performed with the tritium counting window at factory settings (lower = 32 mV, upper = 470 mV) for tritium.

Dynamic counting

The experimental system consisted of a Series 4 HPLC pump (Perkin-Elmer, Norwalk, CT, U.S.A.) connected directly to a FLO-ONE/DR radioactivity monitor. The system did not include an injector or columns. Pump solvent reservoirs A, B, C and D contained water plus tritiated water (50 000 dpm/ml), acetonitrile plus tritiated water (50 000 dpm/ml), water and acetonitrile, respectively. A flow-rate of 1 ml/min was used for all experiments. The time required for changes in solvent composition at the pump to be evident at the detector was determined by switching from acetonitrile (D) to acetonitrile containing tritiated water (B) and noting the time needed to obtain a constant level of radioactivity (4 min). For the detector evaluation experiments, the pump was programmed to deliver the solvent compositions shown in Fig. 1. Solvents in reservoirs A and B were used to maintain a constant concentration of tritiated water and C and D were used for background runs. The detector scintillator was pumped at a flow-rate of 3 ml/min (3:1, scintillator:solvent) and counts were accumulated and printed at 0.2-min intervals. Data collection

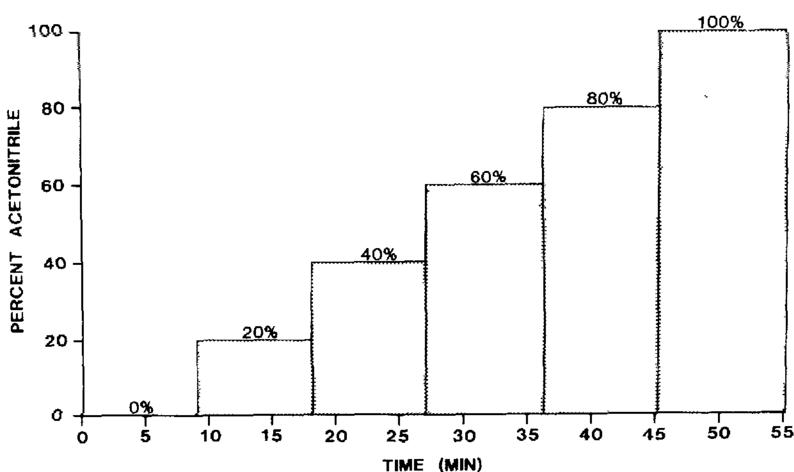

Fig. 1. A step gradient from 0 to 100% acetonitrile. For a change in solvent composition to be evident at the detector 4 min were required. Fractions were therefore counted at 5—10 min after the beginning of each isocratic region.

was initiated at the beginning of the solvent delivery program by a contact closure. Data from a 5-min period (25 measurements) during each interval of constant solvent composition were selected for calculations and comparison. The experiments were performed with the tritium counting window at factory settings (Vide Supra).

Fraction collection

Solvent compositions were programmed for HPLC pump delivery as shown in Fig. 1. Fractions were collected in a fraction collector (Model 328, ISCO, Lincoln, NE, U.S.A.) in 4-ml scintillation vials at 0.2-min intervals with a Model 590-001 flow interrupter valve (ISCO) used to prevent spillage between tubes. Fraction collection was initiated by a contact closure at the beginning of the solvent delivery program. A 3-ml volume of scintillator (25% TRITON-X, 75% Omni-Scint, 4 g/l in toluene, ICN Chemical and Radioisotope Division, Irivine, CA, U.S.A.) were added to each vial and counted for 1 min in a Beckman LS-335 scintillation counter. As with the radioactivity monitor, data from a 5-min period (25 tubes) during each interval of constant solvent composition were used for calculations and comparison.

Calculations

Counts measured by the radioactivity monitor were converted to counts per minute (cpm) by the equation

$$\text{cpm} = \text{counts} \times F/V = \text{counts} \times 8\ \text{min}^{-1}$$

where F = flow-rate through the cell (4 ml/min) and V = volume of the flow cell (0.5 ml). Fractions counted with the scintillation counter were determined directly in cpm.

Counting efficiencies were calculated by dividing the net cpm (average cpm − average background cpm) by the known disintegrations per minute (dpm) in the 0.2-min fractions (10 000 dpm).

The detection limit at each solvent composition was estimated by dividing the standard deviation (S.D.) of the background cpm (n = 25) by the counting efficiency (E), dividing by 0.2 ml to convert the value to dpm/ml, and multiplying by a factor of 3.

$$\text{Detection limit (dpm/ml)} = \frac{[\text{S.D. of background (cpm)}] \times 3}{E \times 0.2\ \text{ml}}$$

RESULTS

Counting efficiency

Frey and Frey [3] determined the counting efficiencies of three HPLC detectors. They found the efficiency of a conventional liquid scintillation counter to be higher than the static efficiencies of the detectors. It has also been noted that the dynamic counting efficiencies of detectors are slightly lower than their static efficiencies [3—5].

In this study, the effect of acetonitrile concentrations on the counting efficiencies of the detector and a conventional liquid scintillation counter was

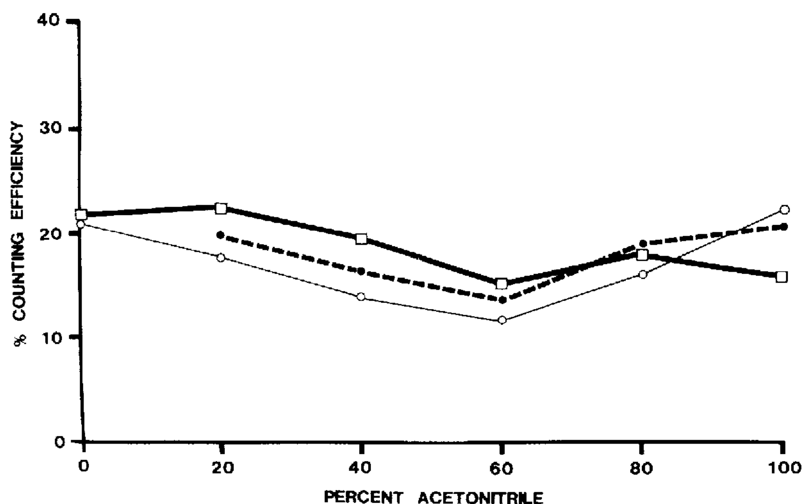

Fig. 2. Counting efficiency as a function of solvent composition determined with 50 000 dpm/ml tritium. (○) Detector (dynamic); (●) detector (static); (□) fraction collection.

examined (Fig. 2). The dynamic efficiency of the detector was slightly lower than its static efficiency except at 100% acetonitrile. This is in agreement with others [3—5]. The efficiency of the liquid scintillation counter was better at low acetonitrile levels but above 80% acetonitrile, the detector's efficiency was superior. The differences observed here may simply be due to the different scintillator fluids used.

The counting efficiency of the scintillation counter was below 25% at all solvent compositions. This low efficiency is primarily due to the acetonitrile that severely quenches and the Triton-X detergent in the scintillator which

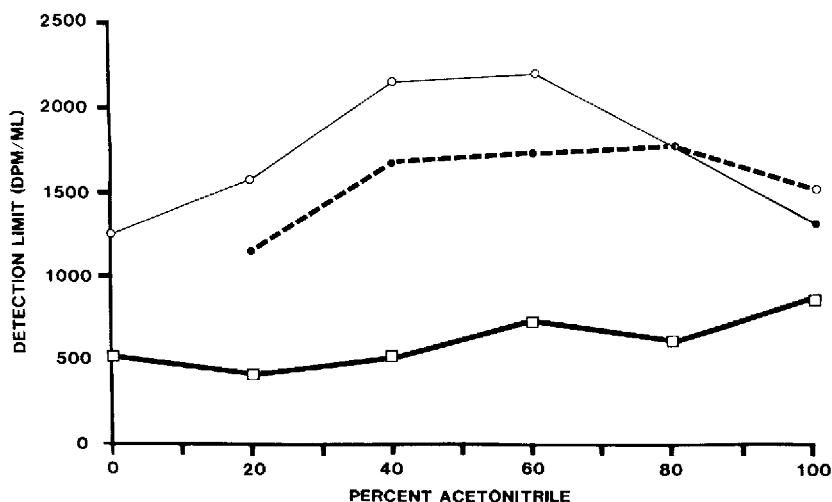

Fig. 3. Detection limits estimated from the variability of the background radiation and the counting efficiency. The variability of the measured background radiation is smaller for the liquid scintillation counter and therefore less radioactivity is necessary to be distinguishable from the base-line. (○) Detector (dynamic); (●) detector (static); (□) fraction collection.

maintains a homogeneous solution for good counting precision but at the same time quenches the fluorescence.

Detection limits

The concentration of tritium necessary to be distinguishable from background counts was considerably less for the scintillation counter than the detector (Fig. 3). The detector's poorer detection limits are mostly attributable to the greater variability of the detector's measured background radiation. This is not unexpected because of the shorter counting time of the detector (0.2 min versus 1 min).

Reproducibility

The usefulness of a radioactivity detector does not depend solely on its counting efficiency. Rather, it is the precision of the measured count-rate that is more critical. If the reproducibility is poor then the resulting chromatogram will be noisy and peak determination will not be straightforward. Due to the random nature of radioactive decay and the frequent sampling intervals and small flow cell sizes required to resolve HPLC peaks, the accumulated counts in the intervals can be small and the error in the estimate of cpm can be relatively large. Inconsistent or incomplete mixing of the scintillation fluid with the HPLC eluate may also contribute to counting error. Only Kessler [4] has examined the reproducibility of a radioactive detector; he reported standard deviations of 2—5% for sample peaks containing 15 000—100 000 dpm. However, good precision with a large number of counts accumulated over the entire peak is not unexpected.

We found that the ability of the detector to precisely measure a quantity of tritium in a flowing stream was better than that obtainable by fraction collection and counting. When 50 000 dpm/ml was pumped at 1 ml/min through the

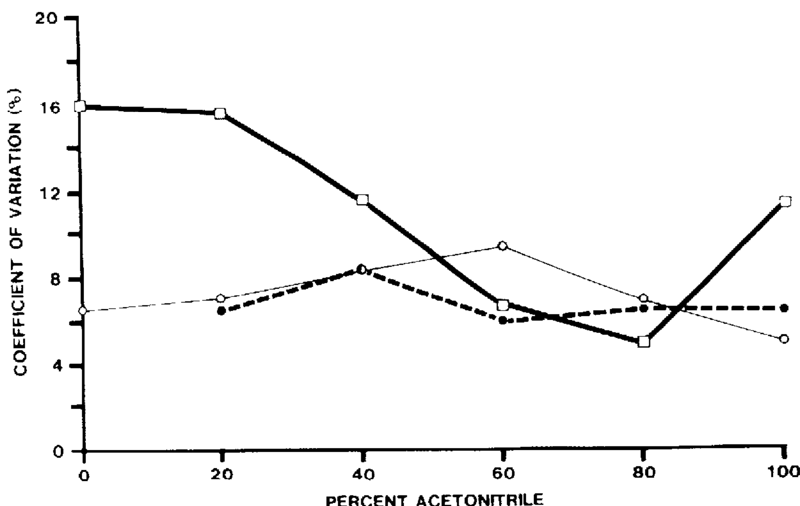

Fig. 4. Counting reproducibility of 50 000 dpm/ml tritium pumped at 1 ml/min directly through the detector or to a fraction collector followed by scintillation counting. (○) Detector (dynamic); (●) detector (static); (□) fraction collection.

detector, the coefficient of variation in 0.2-min counting intervals ranged from 5.1 to 9.5% and was relatively constant across the spectrum of solvent compositions (Fig. 4). Using the alternative approach of collecting fractions every 0.2 min and counting with a scintillation counter, the variability was considerably higher at solvent compositions below 50% acetonitrile (Fig. 4). The coefficients of variation at 0, 20, and 40% acetonitrile were 15.9, 15.7 and 11.6%, respectively.

There is no doubt that this imprecision stems from the variability of the collected volumes in the fractions. At lower acetonitrile concentrations the surface tension is high and drop size is large. Since collection is on a time basis, the tubes do not all receive the same number of drops and the difference of a single drop results in a significant difference in volume and radioactivity. At higher organic solvent compositions, the drops are smaller, more frequent and the difference of a single drop is not as significant resulting in lower coefficients of variation.

DISCUSSION

The innate weakness of on-line monitoring of radioactivity in solvent streams is the short residence time of the radioactive elements in the detector cell. Unlike other detection systems which involve the measurement of an essentially continuous signal (generally electromagnetic radiation or an electrical current), the measurement of radioactivity relies on the counting of relatively infrequent and random discrete events to estimate the concentration of a particular unstable isotope. Therefore, the shorter the residence time of the sample in the detector cell, the fewer counts accumulated and the greater is the probable error in the estimated concentration. For this reason, we suspected that fraction collection and counting might still be a better approach since counting time is less of a limiting factor even though it is labor-intensive.

However, this study revealed that this advantage is overshadowed by the difficulty in obtaining fractions of small equal volumes in short time intervals. Many reversed-phase HPLC separations are performed at organic solvent compositions of less than 50% and this is where the variability is the greatest. A likely result of the collection and counting procedure therefore is a noisy radioactivity peak profile with splitting of some peaks. We have in fact observed this phenomenon when separating small radiolabeled peptides that elute at around 20% acetonitrile. The detector on the other hand has a lower and more constant variability at all solvent compositions and does not exhibit this problem.

We have experienced a minor problem with the radioactivity detector though which we have not seen mentioned in the literature. When examining radiolabeled luteal peptides from culture media by HPLC, we observed a gradual increase in the background radiation. Presumably this was due to the sorption of peptides onto the PTFE flow cell of the detector. On the advice of the manufacturer, we have found that this can be effectively decreased by pumping a concentrated solution of Terg-A-Zyme detergent (Alconox, New York, NY, U.S.A.) through the cell followed by methanol and then by the scintillator.

Finally, we would like to comment on methods for presentation of radioactivity chromatograms. The raw data from a detector are in the form of counts

accumulated in a specified counting period at a particular flow-rate through the cell. Some authors have plotted counts on the y-axis, but they sometimes leave out details such as counting time, scintillator flow-rate, or flow-cell volume, so that calculation of the quantity of radioactivity (total cpm or dpm) in any particular peak is not possible. Not only is it helpful to the reader to be able to do quantitative calculations on these data, but it is also important to the researcher in order to do subsequent evaluation of the peak components using other techniques such as electrophoresis, amino acid sequencing, and size exclusion chromatography.

Another approach has been to first convert the counts to cpm by the equation

$$\text{cpm} = \text{counts} \times F/V$$

where F = total flow-rate and V = volume of the flow cell. Estimation of the total cpm in a peak can then be more readily calculated:

$$\text{total cpm} = 1/2 \times \text{peak height} \times \text{peak width/counting time}$$

where peak height is in cpm and peak width in units of time. Unfortunately though, the counting time is sometimes omitted by authors making this calculation impossible.

A third approach which is presently favored by us, is to use units of concentration (cpm/ml or dpm/ml) on the y-axis:

$$\text{cpm/ml} = \text{cpm/(counting time} \times \text{column flow-rate)}$$

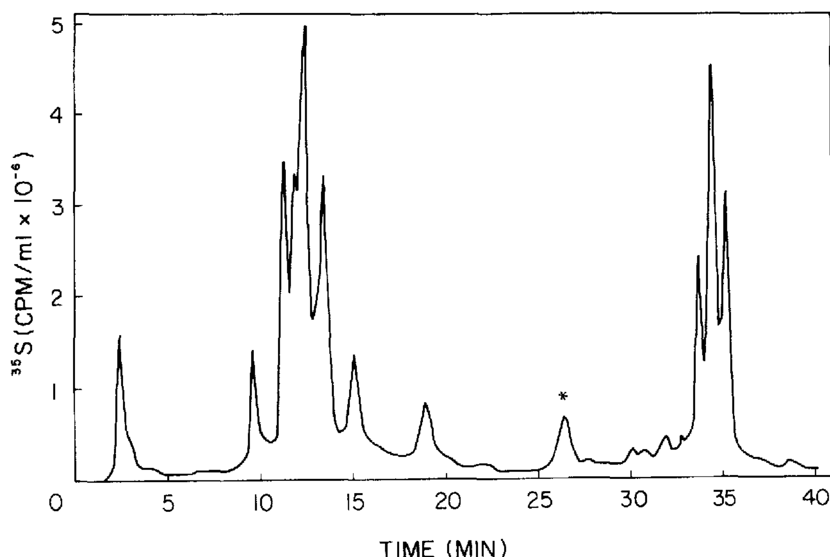

Fig. 5. HPLC profile of radiolabeled peptides in medium from an incubation of bovine ovarian tissue. The values plotted on the y-axis (cpm/ml) were calculated from the measured counts, total flow-rate (4 ml/min), flow-cell volume (0.5 ml), counting time (0.2 min) and column flow-rate (1 ml/min) as described in the text. The quantity of radioactivity in the peaks can be estimated from the peak heights, widths and flow-rate when presented in this format. The peak designated by an asterisk contains approximately 450 000 cpm (1/2 × 570 000 cpm/ml × 1.6 min × 1 ml/min).

The quantity of radioactivity in peaks can then be estimated:

total = 1/2 × peak height × peak width × column flow-rate

where peak height is in cpm/ml or dpm/ml, peak width in min, and flow-rate in ml/min. Since the detection limit at a particular flow-rate and counting time can also be expressed in concentration units (Fig. 3), the quantity of radionuclide necessary for detection in a chromatographic peak is calculated in the same manner:

peak detection limit = 1/2 × detection limit × peak width × column flow-rate

An example chromatogram with calculations is shown in Fig. 5.

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