Continuous β-activity measurement in HPLC eluates

EDWARD RAPKIN

IN/US Systems, Inc.
Pine Brook, NJ, USA

Early references to the measurement of β-activity in flow are found in the mid-1950s. Laboratory adaptations of single-photomultiplier tube (PMT) liquid scintillation counters with solution flowing through, around, or over scintillating plastic gave unsatisfactory results but promising indications. The background level of these single tube counters, largely PMT noise, was excessively high. In addition, chemiluminescence and phosphorescence often contributed more counts to the result than did the actual sample radioactivity. Equally unsatisfactory, the scintillating plastic - beads, tubing, grooved blocks, films - was both a poor scintillator and the surface-to-volume ratio was low resulting in low counting efficiency. With relatively few counts produced and with backgrounds high, for continuous-flow detection to be useful, something better was needed.

COINCIDENCE COUNTERS

Even before 1960, the counter problem was largely solved with the introduction of coincidence-type counters with two PMTs examining the sample. Only if both PMTs pulsed simultaneously could a count be recorded. Coincidence counting masked PMT noise and made chemiluminescence and phosphorescence more manageable. As a result, single tube counters - both for discrete samples and for continuous-flow - have totally disappeared from the market.

Since my first papers (1,2) describing prototype commercial instrumentation for continuous-flow measurement of β-activity, the equipment has undergone continued improvement. Our first radiochromatography monitor (1959) was a modified liquid scintillation counter with a pair of horizontally-opposed 2" diameter PMTs viewing a tubular Kel-F cell about the size of a cigarette through which eluate flowed. The cell, packed with crystalline anthracene (blue-violet fluorescence) having a relatively large surface area, was a potential improvement over the previous scintillating plastic devices. Radioactive decay produced photons, sometimes in sufficient numbers - a function of the energy of each individual β-particle - to pulse both PMTs at the same time. Those events resulting in coincidences provided a measure of radioactivity.

Forty years later, general principles haven't changed but the equipment has. Early on, even before there was HPLC, there was recognition that quantitation required uniform eluate flow. That restricted radiochromatography to use with amino acid analyzers, the only chromatography equipment, circa 1960, to routinely include metering pumps. But still, our detector wasn't really useful. Amino acid analysis was a lengthy procedure; at first 48-hour chromatography runs were required for a complete analysis but later improvements reduced the time to perhaps 6 hours. With low efficiency and peaks spread over many minutes, but with background continuous,

radiochromatography did not generate much interest.

Looking back, it is obvious why this was so. Long runs meant solid scintillator and at that time solid scintillator meant crystalline anthracene. Though mixing column eluates with liquid scintillator was known to give greater light output, hence better results (3), the cost of liquid scintillator for long runs was prohibitive. And anthracene, as a scintillator, was not a good choice. Inherently a good scintillator, anthracene is in reality a poor material for a flow-through system. It is soluble in organic solvents, thus preventing their use in chromatography as potential mobile phase components. Further, a surprising variety of substances adhere to anthracene and, if labeled, contaminate the cell and elevate the background. It is not easily wet with water. Its crystals cannot be tightly packed and lower energy β-particles give up their energy to the mobile phase before they can interact with the scintillator. Of the two β-emitters of greatest interest, one, ^{3}H with an E_{max} of 18.6 keV, was nearly unmeasurable with anthracene. The other, ^{14}C with an E_{max} of 156 keV, was measured with just 10-20% counting efficiency.

HPLC

About 1965, liquid chromatography was dramatically changed with the appearance of HPLC. The 6-hour amino acid analysis was now completed in one-hour or less, as was almost every other separation; HPLC was widely applied rather than being restricted to a few specialized situations. In the bio-medical research laboratory HPLC quickly became the preferred analytical separation method, after a few years supplanting gas chromatography. This was particularly true in both plant and animal metabolism studies. And HPLC and radiochromatography seemed to be made for one another.

Most importantly, for every HPLC run there was an accurate high-pressure metering pump for the mobile phase. One-hour became a de facto time limit; if a chromatogram could not be completed within an hour, alter the conditions so that it can be. With one-hour runs, and with mobile phase flow at 1 mL/min, radiochromatography with liquid scintillator - 3, 4, possibly 5 mL/min - became practical, with total consumption of no more than 300 mL/run, and usually less.

SCINTILLATORS

At more or less the same time, three insoluble inorganic scintillators were introduced. Calcium fluoride, yttrium silicate, and scintillating glass had different properties, but all were superior to anthracene. They were, of course, much less soluble in organic solvents. They were amenable to crushing and sieving, something which could not be done with anthracene, and uniform particle size combined with high pressure HPLC pumps allowed the use of finer particles, leading to smaller interstitial volumes and higher counting efficiencies (1-3% for ^{3}H, 40-70% for ^{14}C). Though they bind to different substances and so become contaminated, having a selection greatly improves the odds that at least one will be satisfactory for a particular requirement.

And, over the years, problems with liquid scintillator have largely been eliminated. Improved formulations offer greater fluidity and higher counting efficiencies (20-40% for ^{3}H, 60-80% for ^{14}C) even at scintillator:mobile phase ratios as low as 3:1. Additives reduce chemiluminescence and phosphorescence while facilitating the uniform dispersion of buffers, even those with high salt content, in relatively small amounts of scintillator. Similarly, insoluble scintillators have been improved, most notably by silanizing to eliminate binding sites, thereby reducing the risk of contamination.

MEASUREMENT CELLS

Along with scintillators, cells have been perfected (Figure 1). Most cells, either for liquid scintillation counting or packed, are now made in the form of a coil using narrow-lumen thin-wall transparent/translucent Teflon or Tefzel tubing. Cell volumes range from 10 to 2000 µL but are usually from 250 to 1000 µL. Larger cells are employed for low-activity counting and smaller ones are used when activity levels are high or peaks are closely spaced.

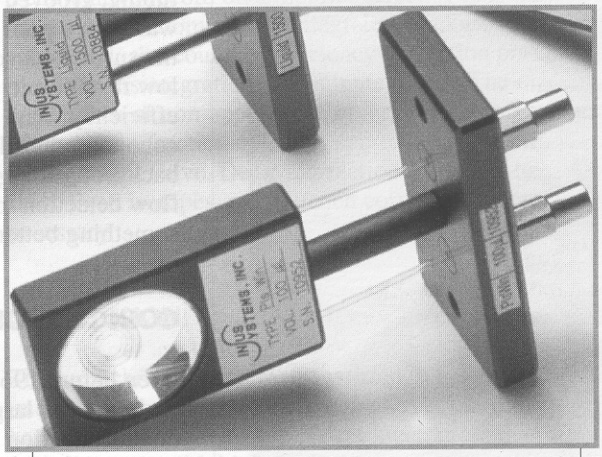

Figure 1 - Modern counting cell with packed scintillator. The cell coil is in a reflective cylinder to maximize light collection. There are no internal tubing connections in order to eliminate potential leakage sites

Such coils typically withstand backpressures of 100-125 p.s.i. but, should that be inadequate for some applications, medium- and high-pressure cells are available.

Especially for ^{3}H measurement, the counting efficiency for solid scintillators is enhanced by the use of finer particle size. Scintillation properties are

not improved, but rather finer particles pack more tightly thereby reducing the distance through which a β-particle must travel before interacting with scintillator. The maximum energy β-particle from ^{3}H decay (18.6 keV) is completely absorbed by 6 µL of water (4). Thus, with tighter packing the probability of useful interaction between the β-particle and scintillator is increased and with that, so is counting efficiency.

Unfortunately, finer particles also mean increased backpressure, thereby risking cell rupture. This has been countered by constructing cell coils of heavier wall tubing, or even encapsulating the coil in UV-transparent resin. Not wholly without penalty, both reduce light output as the heavier wall or the resin each have some absorbency. Nevertheless, the net effect is positive and medium- (to 1000 p.s.i.) and high-pressure (to 3000 p.s.i.) cells for solid scintillator are an important part of the flow-through detector picture. In addition to their application for ^{3}H counting, which for that purpose remains secondary to the use of liquid scintillators, today medium- and high-pressure packed cells are widely used for detectors that are directly on-line to mass spectrometers.

PHOTOMULTIPLIERS

But, it is the instrumentation itself that has experienced the most improvement. Notably, modern instruments outperform earlier ones, with higher counting efficiencies and lower backgrounds. They are smaller than ever, partly in recognition of the crowded conditions of many laboratories, but mainly resulting from the availability of smaller PMTs, which actually give superior performance to larger ones of the historical past. Since the PMTs have more effect on the final design outcome - performance, size, physical configuration, electronics, cell construction - than any other aspect of the system, they merit further discussion.

One must bear in mind that the flow-through detector cell has a volume of perhaps 1000 µL, rarely a little more and frequently substantially less. Though flow-through detectors began as an adaptation of liquid scintillation counting equipment with 2" PMTs used for the examination of 20 mL sample vials, there is no logical reason to have continued with exactly the same PMTs for cells that are so much smaller. This argument is reinforced by several considerations.

As compared to a 20 mL counting vial, the small flow-through measurement cell presents a small target for ambient radioactivity, cosmic rays, and other external radioactive contributors to background. The principal component of the "background" of a flow-through detector is PMT "crosstalk", caused by light internally generated in either of the two tubes and incident on both photocathodes, resulting in simultaneous pulsing and a false count. The largest contributor to this light is Cerenkov radiation, light produced within the glass envelope of the tube by trace quantities of ^{40}K; a 2" dia PMT has about four times the ^{40}K content of a $1^{1/8}$" tube, hence four times the Cerenkov and four times the crosstalk.

If that is not sufficient reason to employ smaller tubes, there are others. The same shielding thickness is achieved with substantially less weight in lead, leading to a smaller detector housing and ultimately a smaller cabinet. The $1^{1/8}$" PMTs operate at 500-800 V less than 2" tubes. As a result, they are less noisy, yet they actually have more sensitive and more uniform photocathodes. And, as an added bonus, and a substantial one at that, they are less expensive.

MODERN INSTRUMENTATION

Modern instruments are also more self-contained (Figure 2). The smaller detector assembly allows room for internally mounting the scintillator pump, a mixing tee, and the associated plumbing. Most systems used with liquid scintillator also include an input splitter,

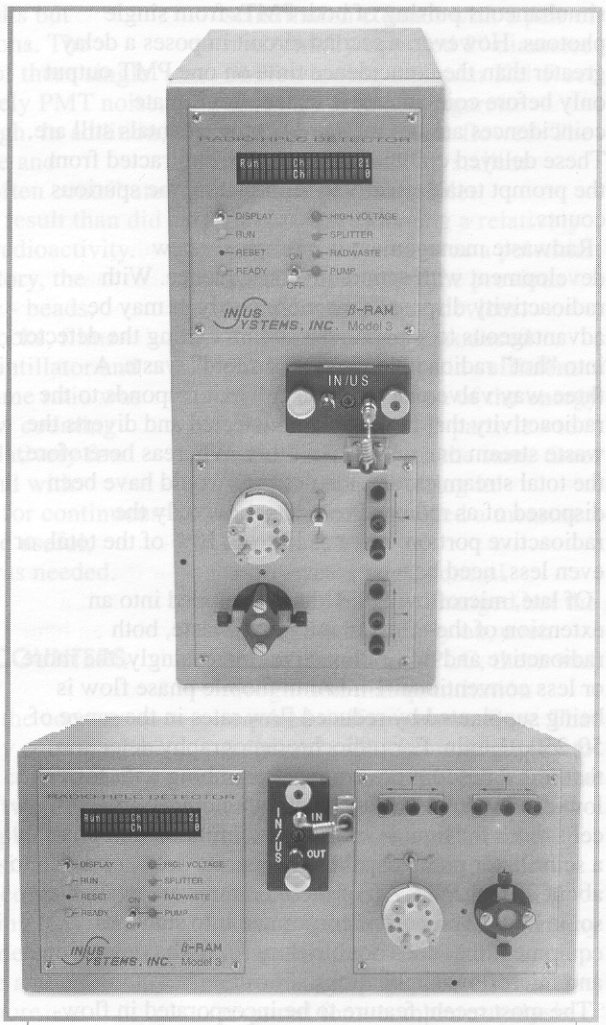

Figure 2 - IN/US β-RAM Model 3, introduced in 2000. Entirely self-contained, coincidence detector and electronics, scintillator pump, splitter and RadWaste valves, and microprocessor are in one cabinet capable of vertical or horizontal placement

which allows part of the eluate to be diverted around the system. Useful when it is necessary to collect a portion of the HPLC eluate free of liquid scintillator, the splitter either takes the form of a fixed tee or, more often, a fast-acting three-way valve which alternately directs eluate first to the detector then around it. In preparative work, the splitter finds a place when activity levels are so high as to swamp the detector.

Chemiluminescence and phosphorescence, are due to single photons produced by spurious processes including external light stimulation. They offer the potential for false counts. With liquid scintillator, additives sometimes suppress the phenomena. When rates are moderate, fast (<100 nsec) coincidence circuitry reduces the possibility that single photons will result in both PMTs pulsing within the resolving time. When additives fail and rates are excessive, something else is needed to make flow-through measurement feasible. Modern flow-through systems incorporate some form of delayed coincidence counting (5). Prompt circuits record all coincidences, from real decay events, from background, and from "accidentals" derived from simultaneous pulsing of both PMTs from single photons. However, a second circuit imposes a delay greater than the coincidence time on one PMT output only before coincidence is tested. Legitimate coincidences are not measured but accidentals still are. These delayed coincidences are then subtracted from the prompt total, effectively eliminating the spurious counts.

Radwaste management is a relatively new development with economic consequence. With radioactivity disposal ever more costly, it may be advantageous to separate the stream exiting the detector into "hot" radioactive waste and "cold" waste. A three-way valve placed at the cell exit responds to the radioactivity that has just been detected and diverts the waste stream one way or the other. Whereas heretofore, the total stream exiting the detector would have been disposed of as radioactive waste, now only the radioactive portion, often as little as 10% of the total, or even less, need be.

Of late, microflow HPLC has developed into an extension of the effort to minimize waste, both radioactive and non-radioactive. Increasingly, the more or less conventional 1 mL/min mobile phase flow is being supplanted by reduced flow rates in the range of 50-250 µL/min. For radiochromatography detection, that translates to more compact plumbing with fewer, low-dead volume, fittings, correspondingly smaller cells, both for liquid- and solid-scintillator, and possibly a scintillator pump capable of accurate delivery down to about 200 µL/min. All of these features are available; some have even been incorporated into standard equipment that does double-duty for both conventional and microflow applications.

The most recent feature to be incorporated in flow-through detectors has been a 10-port injection valve with two calibrated sample loops. Used to allow direct introduction to the measurement cell of known quantities of standards and other samples that are not subjected to chromatography, the injector valve simplifies counting efficiency determination for single and dual isotope measurement. Recovery studies are simplified by first injecting a composite sample via one of the loops and then reporting each peak as a percent of the total after that same sample has been subjected to HPLC. And whether or not there is "quenching" - performance degradation resulting from gradient change during chromatography - is established by injecting the same standard at the beginning, end, and possibly one or more times during a run to observe changes.

SOFTWARE

Though many detectors output analog information to the software that is controlling the HPLC unit, data processing has become an integral part of most flow-through systems. Early systems fed count-rate output to strip-chart recorders; digital electronics were too slow to record data taken on the fly. But, over the years, data treatment has undergone considerable change. The latest systems incorporate microprocessors and most provide second-by-second digital output to Windows-based personal computers. Some systems output directly to an Excel worksheet where counting data may be merged with results from other measurements of the same components.

Modern software tends to be comprehensive. First, it runs the instrument, setting counting windows, scintillator flows, split, sometimes PMT high voltage, run length, etc. Then, it receives incoming data and displays it, often numerically point-by-point and also as a cumulative record on a monitor screen. Peaks may be manually or automatically integrated, background subtracted, and counting efficiency correction provides results in disintegrations per minute (dpm). The appearance or non-appearance of a peak can control the advance of a fraction collector and/or the radwaste management valve. Decay correction is applied to short-lived isotopes, as is quench correction when necessary.

CONCLUSION

The radiochromatography flow-through detector has undergone continuous improvement over the years it has been on the scene. At first not much more than a curiosity, now with high throughput, greater sensitivity, and sophisticated data treatment possibilities, it has become an indispensable tool of metabolism research.

REFERENCES

1. E. Rapkin and L.E. Packard; U.S.A.E.C. Technical Document 7612, 1961, p.216
2. E. Rapkin and J.A. Gibbs; *Nature* **194**, No. 4823, 34 (1962)
3. H.W. Scharpenseel and K.W. Menke; in: Tritium in the Physical and Biological Sciences; I.A.E.A., Vienna, 1962, Vol.1, p.281
4. K.J. Hufsteder and H.T. Wilson; *J. Fusion Tech.* **21** 446 (1992)
5. P. Giraud, C. Palais and E. Rapkin; U.S. Patent 4,031,392 (1977)