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Direct and reverse flow-through heterogeneous scintillation counting of radiolabelled amino acids using post-column solvent segmentation

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SUMMARY

A method for flow-through heterogeneous scintillation counting of ^{14}C-labelled amino acids is described. It is based on post-column solvent segmentation of the aqueous column eluate with hexane before the eluate enters the flow cell of the radioactivity monitor. Using yttrium silicate scintillator granules of 60–80 μm diameter, counting efficiencies of 0.71 (^{14}C) and 0.01 (^{3}H) are obtained. Segmentation of the column eluate allows the temporary storage of the column eluate in a capillary storage loop without additional band broadening and, after the separation is completed, re-introduction of the segmented stream through the radioactivity monitor at reduced flow-rate so as to increase the sensitivity of flow-through radioactivity detection.

INTRODUCTION

Since flow-through radioactivity detectors became commercially available, attempts have been made to overcome the inherent drawback of flow-through radioactivity counting of high-performance liquid chromatographic (HPLC) column eluates, *i.e.*, the coupling of a fast separation with a slow counting step[1–4]. Our group developed a method for flow-through liquid scintillation counting (LSC) of aqueous HPLC column eluates based on the use of a water-immiscible liquid scintillator[3,4]. Before entering the radioactivity detector, the column eluate is extracted on-line with the liquid scintillator (direct counting mode). In addition, the creation of a segmented pattern allows the storage of the complete chromatogram in a capillary storage loop while suppressing extra-column band broadening. When the separation is complete, the contents of the storage loop can be re-introduced into the radioactivity detector at low flow-rates (reverse counting mode) to increase the counting time and, hence, the

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sensitivity of flow-through counting relative to the direct counting mode. In this way, counting times of over 10 min can be applied in favourable cases[4]. In order to reduce the analysis time of the reverse counting mode, regions of interest (RoIs) can be defined. Only during these RoIs is the flow-rate decreased while the remainder of the segmented stream is transported through the detector at higher, *i.e.*, conventional flow-rates.

With this principle, the counting efficiency of low- to medium-energy beta emitters was found to be determined primarily by the mobile phase composition and the extraction yield of the analytes from the aqueous into the organic (scintillator) plugs. The proposed method is not applicable in situations where the analytes cannot be extracted efficiently. This applies to *e.g.*, amino acids, peptides, sugars, nucleic acids and catecholamines.

Since in heterogeneous (solid) scintillation counting (HSC) the counting efficiency is determined by the type and packing density of the scintillator granules only[5], it is of interest to study the combined use of post-column segmentation and flow-through HSC. This alternative could widen the range of application of reverse counting to include non-extractable analytes. In principle, any water-immiscible solvent can then be used for segmentation of the aqueous column eluate.

The approach was first studied by van Nieuwkerk *et al.*[6] in the determination of radiolabelled amino acids. They used a cylindrical, 18-μm yttium silicate-packed glass cell. Although transport of purely aqueous or hexane streams posed no problems, it was found that transporting hexane-segmented aqueous streams through the cell led to a significant increase in the pressure drop across the cell and, finally, cell breakage, probably caused by clogging of the packed cell with particulate matter present in the aqueous phase.

In this study, solvent segmentation was combined with reverse flow-through HSC for the determination of radiolabelled polar analytes, using a commercially available radioactivity monitor equipped with an yttrium silicate-packed cell. Before use, particulate matter was removed from all aqueous solvent mixtures by filtration over a 0.2-μm membrane filter. Relatively large (60–80-μm) scintillator granules were used.

EXPERIMENTAL

Apparatus

A schematic diagram of the set-up used is given in Fig. 1. The column liquid chromatographic system consisted of a Model 114M solvent-delivery module (Beckman, Fullerton, CA, U.S.A.), a Model 7126 six-port injection valve (Rheodyne, Cotati, CA, U.S.A.), a cartridge column holder (Brownlee Labs., Santa Clara, CA, U.S.A.) and a Uvikon variable-wavelength detector, equipped with a Model LCD 725 8-μl flow cell (Kontron, Zürich, Switzerland). A Model P-500 syringe pump (Pharmacia, Uppsala, Sweden) was used to deliver the post-column solvent. Mixing of the two streams took place in a 0.25 mm I.D. T-piece (Valco, Houston, TX, U.S.A.). The combined stream was led through a Ramona-5-LS HPLC radioactivity detector, equipped with a 0.50-ml cell (empty volume after packing), constructed from spirally wound PTFE capillary and packed with 60–80-μm yttrium silicate granules (Raytest, Straubenhardt, F.R.G.). After radioactivity detection, the segmented

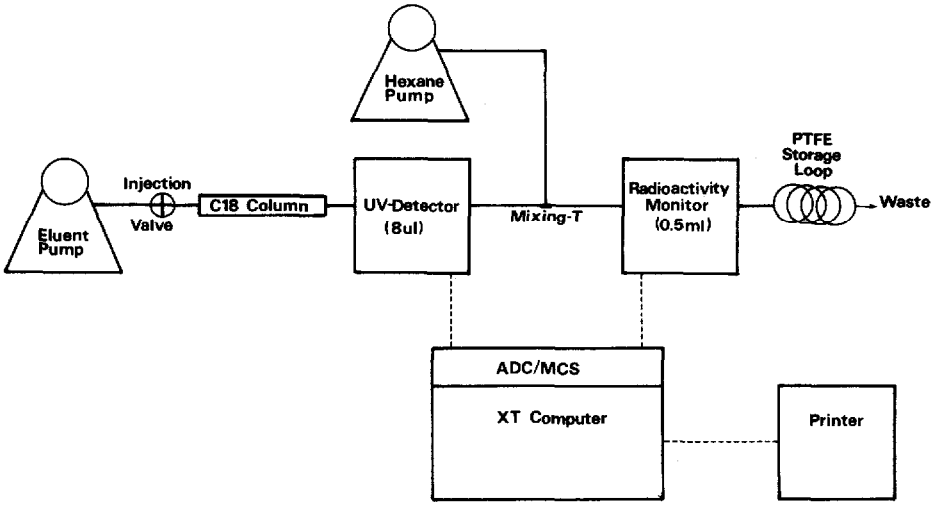

Fig. 1. Schematic diagram of the set-up used for HPLC with flow-through direct heterogeneous scintillation counting (HSC).

stream was stored in a PTFE capillary storage loop (0.8 mm I.D., wall thickness 0.4 mm).

Data acquisition and processing of the analogue (UV) and digital (radioactivity) signal was based on a commercially available software package (Radio-Chromato-Graphic-System, version 10.8-MCS, Raytest). This package consists of a triple trace standard program (IM 2313), run-time peak search/fraction collector control (IM 2303), auto-injector control (IM 2304), quench correction (IM 2310) and manual or fully automatic evaluation (IM 2310). It runs on a Tulip PC compact 2 computer with hard disk (Tulip Computers, 's-Hertogenbosch, The Netherlands), equipped with a ADC/multiscaler card for simultaneous data acquisition of one analogue input (configured for up to 100 mV) and two digital (TTL-pulse) inputs. Hard copies were provided by a Model LC-10 multi-front printer (Star Micronics, Nakayoshida, Japan).

Flow-through counting efficiencies, E, were calculated according to

$$E = C_n(F_e + F_s)/[V_d DPM(s)] \quad (1)$$

where C_n is the net peak area (in counts), F_e and F_s are the flow-rates of the mobile phase and make-up flow, respectively (in ml/min), V_d is the void volume of the packed flow cell of the radioactivity monitor (0.5 ml) and $DPM(s)$ is the absolute activity in the peak (in disintegrations per minute).

Absolute activities in the peaks were determined by the sample channels ratio method. For this, peaks eluting from the HPLC system were collected in 20-ml counting vials and, after addition of 15 ml of a water-miscible liquid scintillator, counted on a Model PW 4701 liquid scintillator counter (Philips, Apeldoorn, The Netherlands). ^3H and ^{14}C calibration standards were obtained from Amersham (Amersham, Bucks, U.K.).

Chromatography

Separations were performed using a 5-μm C_{18}-bonded Spheri-5 column (220 × 4.6 mm I.D.) (Pierce, Rockford, IL, U.S.A.). An injection volume of 23 μl was used throughout this study. Mobile phases were made from HPLC/Spectro-grade methanol (Alltech, Deerfield, IL, U.S.A.) and water, buffered to pH 4.2 with HPLC-grade sodium acetate (trihydrate) (Fisons, Loughborough, U.K.) and glacial acetic acid (J. T. Baker, Deventer, The Netherlands). Sodium 1-hexanesulphonate was used as an additive to the mobile phase (Alltech Europe, Eke, Belgium). Before use, water was deionized using a Nanopure II system (Barnstead, Boston, MA, U.S.A.) and subsequently filtered over a 0.2-μm RC 57 membrane filter (Schleicher and Schüll, Dassel, F.R.G.). Before use, mobile phases were degassed using an ultrasonic water-bath.

Chemicals

L[U-^{14}C]Amino acids and tritiated water were obtained from Amersham. Unlabelled amino acid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and technical-grade hexane (mixed isomers) from J. T. Baker. Pico-fluor-40 (Packard, Groningen, The Netherlands) was used as a water-miscible liquid scintillator for absolute activity determinations.

RESULTS AND DISCUSSION

In a study of the potential of a particular reverse counting mode, the experimental results must self-evidently be compared with those obtained by direct measurements. Both modes of operation are, therefore, discussed below.

Direct measurements

A solution containing five ^{14}C-labelled amino acids and spiked with unlabelled phenylalanine was separated by HPLC with UV and flow-through HSC detection. Phenylalanine is the only amino acid which displays good UV absorption and is commercially available as the ^{14}C-labelled compound. In order to suppress additional band broadening in the radioactivity monitor arising from the relatively large (0.50 ml) cell volume, a make-up flow had to be added after the UV detector. To this end, after UV detection a water-miscible [methanol–water (1:9, v/v)] or, alternatively, an immiscible solvent (hexane) was added to the column eluate. The results are shown in Fig. 2. As can be seen from the phenylalanine signals, the make-up flow allows one to obtain band widths in the radioactivity monitor comparable to those observed in the reference detector.

The counting efficiency, calculated from the ^{14}C peak areas, the absolute activities in the peaks and the mean residence time in the detector according to eqn. 1 varied from 0.70 to 0.72. For tritiated water, a counting efficiency of 0.01 was calculated. The typical pressure drop across the cell was about 0.4 MPa at mobile phase and hexane flow-rates of 1.0 ml/min each. No significant increase was observed during a 2-week period of intensive use. Apart from the relatively large (60–80 μm) scintillator granules, this could be due to the effective removal of particulate matter from the mobile phase before use.

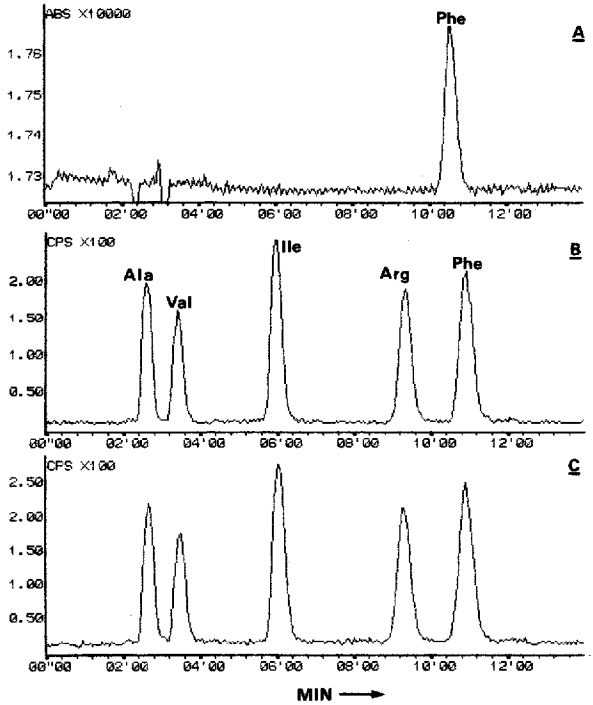

Fig. 2. HPLC of a mixture of radiolabelled amino acids spiked with phenylalanine, with (A) UV detection (220 nm) and (B,C) direct flow-through heterogeneous scintillation counting. Sample, [^{14}C]Ala (350 Bq), [^{14}C]Val (306 Bq), [^{14}C]Ile (544 Bq), [^{14}C]Arg (438 Bq) and [^{14}C]Phe (575 Bq); column, Pierce, 5-μm C_{18} Spheri-5 (220 × 4.5 mm I.D.); mobile phase, methanol–0.05 *M* sodium acetate buffer, pH 4.2 (1:9, v/v) at 1.0 ml/min. In B, a make-up flow of methanol–water (1:9, v/v) was added to the HPLC–UV effluent at 1.0 ml/min via a 0.25 mm I.D. mixing tee. In C, the make-up flow was hexane at 1.0 ml/min.

Reverse measurements

In previous work[6], it was demonstrated that for the storage of segmented aqueous column eluates containing non-extracted analytes, a PTFE storage loop has to be used in order to prevent band broadening via diffusion of the analytes through the aqeous film formed on the inner wall of a stainless-steel tubular capillary. Owing to their low mechanical strength and porosity, PTFE capillaries of 0.4 mm wall thickness have limited pressure resistance, which may hamper their utilization in combination with the transport of segmented streams through packed cells. Fortunately, the conditions encountered in this study posed no problems and flow-rates of up to 1.0 ml/min could easily be employed. In a critical situation, the use of a PTFE-coated stainless-steel capillary may be a good alternative.

In Fig. 3, reverse radiograms of a sample recorded at different flow-rates through the detector are shown; hexane was used as the make-up and segmentation liquid. The additional band broadening can be estimated by comparing the ^{14}C peak widths (in ml) at 10% of the peak height in the direct (not shown) and corresponding reverse radiograms. The ratio of the band widths is a convenient measure for the band

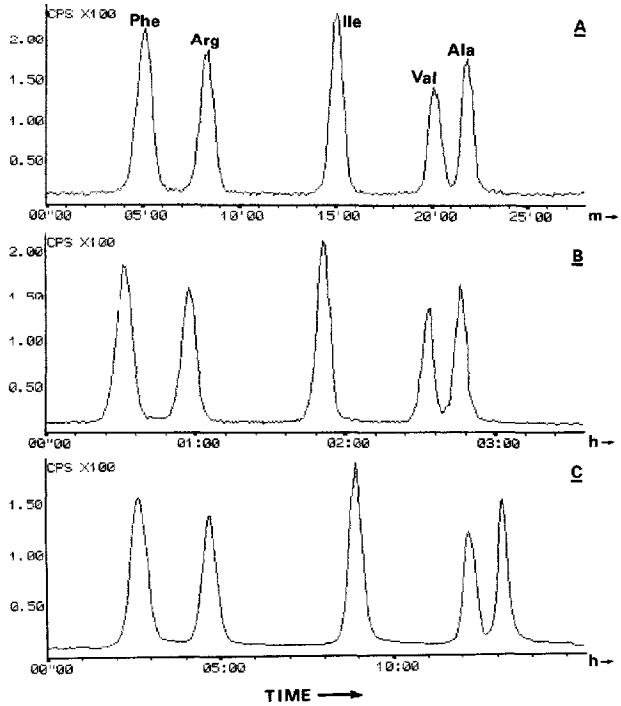

Fig. 3. Reverse radiograms of a mixture of ^{14}C-labelled amino acids, recorded for different counting times, T_d. During the three corresponding direct measurements (data not shown), the HPLC column eluate was segmented with hexane at 1.0 ml/min at the UV detector outlet and stored in a 0.8 mm I.D. PTFE capillary storage loop. After storage of the complete chromatogram, the eluant and hexane pumps were stopped and the outlet of the storage loop was connected to the outlet of the HPLC column. The contents of the loop were then transported through the cell using the eluent pump. The transport flow-rates were (A) 1.0, (B) 0.123 and (C) 0.026 ml/min, corresponding to counting times of 0.5, 4.06 and 19.23 min, respectively. for the HPLC conditions during the direct measurements, see Fig. 2C. Note the different time axes in A (minutes) and B and C (hours).

broadening. In Fig. 4, they are plotted as a function of the counting time. With one exception (alanine), which cannot easily be explained, the amino acids display band broadening ratios between 1.0 (no broadening observable) and 1.2 over the whole range of counting times studied, *i.e.*, for T_d = 0.15–20 min. Obviously, additional band broadening is kept at acceptable levels and certainly is not a function of the counting time.

Table I shows that plots of the net peak area, C_n, *versus* the flow-through counting time of the detector cell, T_d, in the reverse measurements show good linearity for all test solutes over the range T_d = 0.25–4.17 min. The latter counting time corresponds to a flow-rate of 0.12 ml/min through the radioactivity monitor. Note that the slopes of the [^{14}C]amino acid calibration graphs (net peak areas *versus* counting time) differ owing to the different amounts of amino acid in the sample injected onto the column (see Table I).

Deviations from linearity were observed for longer counting times. They are

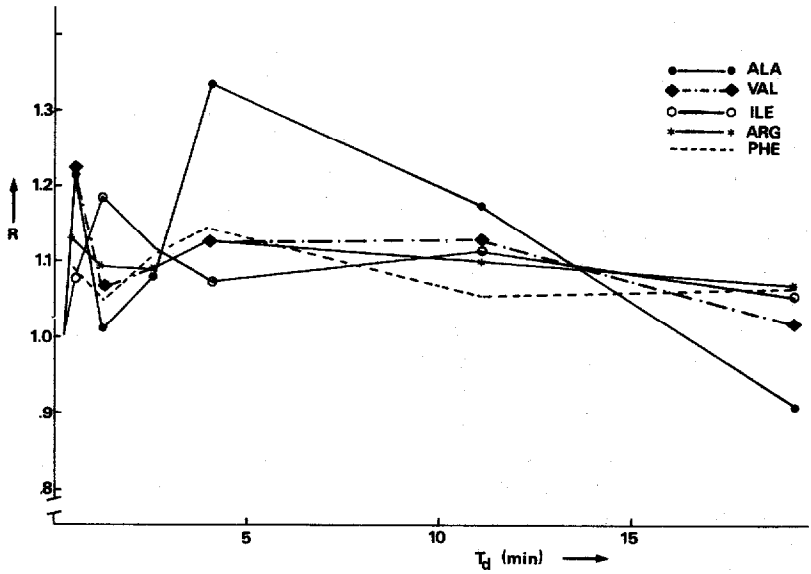

Fig. 4. Band broadening in reverse radiograms relative to that in the corresponding direct radiograms as a function of the counting time, T_d. On the ordinate the ratio, R, of the band widths (measured at 10% of the peak height) from reverse and direct radiograms is given. For HPLC conditions, see Fig. 2C.

best explained from the reduced accuracy in the determination of T_d at the very low flow-rates used for these measurements.

Accordingly, under the conditions mentioned above, a gain factor of about 17 in counting time and, hence, sensitivity is obtained in the reverse counting mode in comparison with the direct counting mode.

CONCLUSIONS

Heterogeneous scintillation counting can be combined with segmentation of aqueous HPLC column eluates to allow both direct and reverse flow-through radioactivity determinations. Additional band broadening is easily kept within acceptable

TABLE I

LINEAR REGRESSION ANALYSIS ON NET PEAK AREAS, C_n (IN COUNTS), *VERSUS* FLOW-THROUGH COUNTING TIME, T_d (IN min)

$C_n = AT_d + B$; T_d ranges from 0.25 to 4.17 min ($n = 5$).

[^{14}C]Amino acid	*Amount injected (Bq)*	*A*	*B*	*R*
Alanine	350	12862	489	0.9998
Valine	306	10509	879	0.9990
Isoleucine	544	18945	1409	0.9992
Arginine	438	15836	1054	0.9996
Phenylalanine	575	19985	1299	0.9996

limits, and flow-through counting times of over 4 min can be used in the reverse mode, which is about a 17-fold increase over direct measurements. As a result, sensitivities comparable to those in off-line liquid scintillation counting can be obtained, yet avoiding most of the disadvantages inherent in the latter technique, such as the loss of resolution in the radiograms, the time-consuming procedure involved in preparing sample vials and data analysis and the high volume of radioactive waste produced. In principle, the total analysis time can be reduced by using flow programming in the reverse mode, as was demonstrated for flow-through liquid scintillation counting[4]. As flow programming of the HPLC pump during a run was not possible with the present set-up, this aspect was not explored.

The limitations of the present principle are inherent in systems using solid scintillator-packed flow cells and are well known from the literature[7]. First, although the yttrium silicate-packed cell has a satisfactory counting efficiency for ^{14}C (E = 0.71), the counting efficiency for ^{3}H was low (E = 0.01) A better performance may be obtained with other types of solid scintillators or by decreasing the scintillator particle size. Second, radiolabelled material may be adsorbed on the scintillator surface and increases in the background count rate can occur after injecting less well defined radioactive samples. In this study, this was observed after injecting an aqueous [^{14}C]leucine sample that had been stored for over 4 years at 4°C. On the other hand, even in this instance, the background count rate could be restored to its original (low) level by flushing the cell with dilute nitric acid. In other words, the introduction of the segmentation/storage principle extends the application range of heterogeneous scintillation counting, without creating additional problems.

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