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QUANTITATION OF RADIOLABELED BIOLOGICAL MOLECULES SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Several techniques are compared for the quantitation of radiolabeled molecules separated by high-performance liquid chromatography (HPLC). The first technique is the direct fraction collection of the HPLC eluent at pre-set times in individual tubes. Aliquots are removed from each tube, placed in scintillation vials, scintillation fluid is added and the radioactivity is counted in a liquid scintillation counter. The second technique is the direct interface (immediate analysis and plotting of data) of the eluent from the HPLC to a flow-through radioactivity detector. The third technique is to split the eluent from the HPLC with a microbore electronic stream splitter (flow/no flow) with a certain percentage directed to the flow-through radioactivity detector and the remainder collected in a fraction collector for further chemical chracterization (gas chromatography–mass spectrometry, recrystallization, further purification). A direct comparison of the chromatograms, radioactivity counting efficiencies and sensitivities for these three techniques is presented.

INTRODUCTION

Radioisotope-labeled compounds have been used routinely to analyze various biological reactions, metabolic pathways and clearance rate studies. In order to quantitate the various resultant radiolabeled compounds the reaction mixture had to be separated by thin-layer chromatography, paper chromatography, column chromatography and other techniques. Once the radiolabeled compounds had been separated, quantitation could be accomplished by one of several methods: paper chromatography (the paper cut into small segments and counted), thin-layer chromatography (the plates being scraped, eluted and counted) or column chromatography (fractions collected and counted). All of these methods were either time consuming (column chromatography) or tedious to count (thin-layer chromatography).

With the development of high-performance liquid chromatography (HPLC), rapid separations of radiolabeled compounds could be achieved[1]. The major problem with applying HPLC to radiolabeled compounds was the accurate quantitation of the radioactivity of each of the peaks as they eluted from the column. Initially, this was accomplished by fraction collecting of tubes at pre-set times, removing an aliquot into

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a scintillation vial, adding scintillation fluid and quantitating in a liquid scintillation counter[2]. This was a very expensive and time-consuming process when several compounds per sample were being separated by HPLC.

Subsequently, a flow-through radioactivity detector, in which a scintillator is bound to a solid support, was developed[3,4]. This technique was used mainly for high-energy β-emitters (^{14}C, ^{35}S, ^{32}P) with counting efficiencies of 10–30% and 0.1–1.0% for ^{3}H. One problem was that this technique could not be used for double-label studies in which both ^{14}C and ^{3}H were present. This paper compares the use of a direct homogeneous flow-through radioactivity detector (HPLC eluent mixed with scintillation fluid) for the quantitation of doubly or singly labeled samples eluting from the HPLC column with the fraction collection and counting method. In addition, a technique for the recovery of a portion of the HPLC eluent and quantitation of the remainder of the sample with a flow-through radioactivity detector was investigated.

EXPERIMENTAL

Apparatus

A Waters (Milford, MA, U.S.A.) HPLC system was employed for all of the studies. This system consisted of a U6K injector, one 6000 A and one M45 high-pressure pump, which constitute the solvent delivery system. Also, a Model 450 variable-wavelength detector, a Model 440 fixed-wavelength (254 nm) detector, a Model 730 Data Module and a Model 720 Data Controller were used. A stainless-steel reversed-phase (3 μm) C_{18} (12% ODS) column (10 cm × 4.5 mm I.D.) (Custom LC, Houston, TX, U.S.A.) was employed for high-resolution HPLC. The HPLC system was also equipped with an in-line pre-column filter. All experiments were performed at ambient temperature.

Chemicals and reagents

High-purify water was obtained by triple glass distillation of deionized water in our laboratory. Glass distilled methanol (Omni Solv-MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.) was used. The steroids (non-radiolabeled) were obtained from Steraloids (Wilton, NH, U.S.A.) and the radioactive steroids from New England Nuclear (Boston, MA, U.S.A.). The buffers and solvents were filtered through 0.45-μm Millipore filters and degassed to avoid bubble formation during use.

Radioactivity detection system

One of the following three methods was employed for the detection of radioactive metabolites resulting from the incubation of tissues with radioactive precursors: (1) interfacing the Waters HPLC system with a Redi Rac Model 2112 fraction collector (LKB, Rockville, MD, U.S.A.), collecting fractions at 0.5-min intervals and counting aliquots in scintillation fluid (Liquiscint; National Diagnostics); (2) direct interfacing of the HPLC with a Flo-One HP or a Flo-One HS (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) flow-through radioactivity detector and using a non-gelling scintillation fluid (Flo-Scint II) at an effluent to scintillation fluid ratio of 1:3 (v/v); pen 2 of the Waters Data Module was set to plot the Flo-One HP signal for ^{3}H, the data being plotted directly as a histogram; the quantitation of both

^{14}C and ^{3}H was effected simultaneously with the results being printed out directly on the Flo-One HP thermal printer; or (3) for comparing the Flo-One HP and the aliquot methods, the eluent from the column was diverted to an electronic variable ratio liquid splitter (Radiomatic Instruments and Chemical Co.) and 50% of the sample was collected at 0.5-min intervals in the Redi Rac fraction collector. The remaining 50% of the sample was counted directly in the Flo-One HP flow-through radioactivitv detector.

Preparation of radiolabeled products

Human and monkey testicular biopsy tissues were incubated *in vitro* with [^{3}H]pregnenolone by a technique described previously[5]. This involves incubation of teased testicular tissue (*ca.* 20 mg) with a saturating concentration of [^{3}H]pregnenolone in 3 ml of incubation medium in an atmosphere of oxygen–carbon dioxide (95:5) at 37°C with constant shaking for 3 h. At the end of the incubation, ^{14}C-labeled recovery tracers and non-labeled carrier steroids were added. The radiolabeled metabolites formed and the remaining substrate were extracted with an organic solvent and dried. The extract was dissolved in 50 μl of methanol and injected into the Water HPLC system using the 3-μm C_{18} (12% ODS) column described earlier[6,7]. The procedure for incubating the interstitial cell cultures with radioactive precursors has been described elsewhere[8].

RESULTS AND DISCUSSION

The problem of quantitation of low-energy β-emitters (^{3}H) and dual-labeled (^{14}C and ^{3}H) radioactive products resulting from biological samples was examined. First, the direct analysis and quantitation of peaks eluting from the HPLC column was examined by the technique of direct fraction collection of the HPLC effluent, removing an aliquot from each tube into a scintillation vial, adding scintillation fluid and counting in a liquid scintillation counter. In order to evaluate this technique a human testicular biopsy was incubated with [^{3}H]pregnenolone. At the end of the incubation, ^{14}C recovery tracers were added in order to correct for the differential organic extraction of the various steroids. The extracted sample was dried, redissolved in methanol and separated by HPLC on a C_{18} column and fractions were collected, aliquots removed and scintillation fluid was added, followed by counting in the liquid scintillation counter, giving a radioactive elution profile (^{14}C and ^{3}H) as shown in Fig. 1b. If the same sample was re-injected and the HPLC effluent coupled directly to the Flo-One HP flow-through radioactivity detector using the liquid mixing system, the elution profile was as shown in Fig. 1a for both ^{14}C and ^{3}H.

The direct chromatogram of ^{3}H from the Flo-one HP as plotted on pen 2 of the Waters data module is shown in Fig. 2. It can be ascertained from the three profiles that the number of peaks, relative peak heights and elution times are almost the same for each chromatogram. It is also apparent that by using the liquid mixing cell in the flow-through radioactivity detector both ^{14}C and ^{3}H can be quantitated in the same sample, with the cross-over of ^{14}C into the ^{3}H being corrected automatically. By using the direct interface of the Flo-One HP flow-through radioactivity detector, both ^{14}C and ^{3}H peaks can be quantitated as the peaks are eluted from the HPLC column without waiting as in the time-consuming and expensive fraction collection and

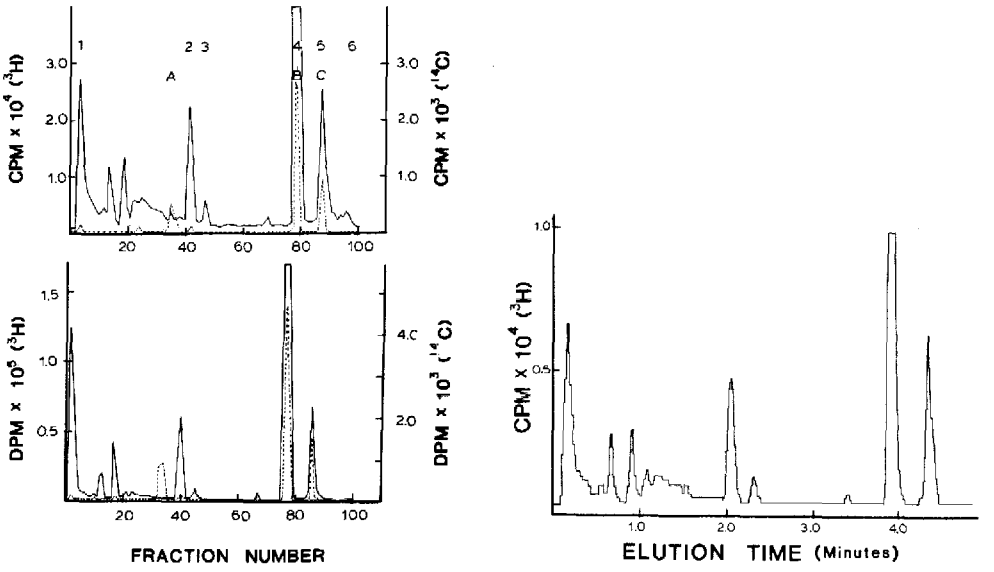

Fig. 1. HPLC of human testicular biopsy specimen (Sweden) incubated with [^{3}H]pregnenolone[5]. At the end of the incubation ^{14}C-labeled steroids were added as recovery steroids and analyzed by HPLC using the conditions in ref. 6. A 3-μm C_{18} (12% ODS) column 10 × 0.62 cm I.D. was used, eluting with a linear gradient of methanol–water from 50% to 100% methanol in 30 min. The lower panel is a plot of the *fraction collector number (0.5 min per fraction) vs. dpm per fraction* for a 10-μl sample injected (total volume = 200 μl). The upper panel shows the direct analysis of injected duplicate 10-μl samples using the Flo-One HP radioactivity detector. The column effluent was mixed in a 1:3 (v/v) ratio with Flo-Scint II in a specially designed mixing tee in the flow-through radioactivity detector. The total combined flow-rate of scintillation fluid and effluent was 8.0 ml/min. The data were printed out at 0.5-min intervals for comparison with the fraction collection and scintillation counting method. The broken line represents ^{14}C and the solid line ^{3}H. The numbers (1–6) above the peaks represent the ^{3}H peaks and the letters (A–C) the ^{14}C peaks analyzed for counting efficiency in Table I.

Fig. 2. The actual (^{3}H) of the sample counted by the Flo-One radioactivity detector as plotted by the Waters Data Module pen 2. The time scale of the actual plot is 0.50 cm/min chart speed and the ^{3}H cpm values are 10,000 cpm full-scale. Chromatographic conditions as in Fig. 1.

scintillation counting method. Next, the efficiency of counting both ^{14}C and ^{3}H for these samples should be considered. Table I illustrates the counting efficiency for the direct radioactivity detector method (40% for ^{3}H and 75% for ^{14}C). The counting efficiency was only slightly reduced in the flow-through radioactivity detector method but the analysis time and the cost per sample were greatly reduced[9].

Even with all of the aforementioned advantages, the one major disadvantage of using the flow-through radioactivity detector is that the eluent is mixed with scintillation fluid. Once the sample has been mixed with the scintillation fluid it is difficult to characterize further (gas chromatography mass spectrometry, recrystallization) or purify the compounds separated by HPLC. A simple method for partially solving this problem is to use a microbore electronic (flow/no flow) stream splitter situated after the last HPLC detector but before the flow-through radioactivity detector. Using this technique a portion (1–99%) of the HPLC column effluent can be diverted to a

TABLE I

COUNTING EFFICIENCY FOR HUMAN TESTICULAR BIOPSY SAMPLES

*Peak**	*Isotope*	*Flo-One HP (cpm)***	*Fraction collection (dpm)****	*Efficiency (%)*
1	^{3}H	78,322	222,990	35.1
2	^{3}H	52,589	117,860	44.6
4	^{3}H	446,609	1,024,640	43.8
5	^{3}H	65,994	150,650	43.8
A	^{14}C	2058	2639	68 §
B	^{14}C	7545	9929	76 §
C	^{14}C	2338	3117	75 §

* See Fig. 1.
** Cpm calculated in scalar mode.
*** Dpm corrected by liquid scintillation counter.
§ Corrected for crossover of ^{14}C into ^{3}H channel.

fraction collector with the remainder being analyzed by the flow-through radioactivity detector. By using this method only a portion of the eluent needs to be used for quantitation of the radioactivity of the sample, the remainder being collected for further analysis of characterization. The electronic stream splitter was used to analyze

TABLE II

COUNTING EFFICIENCY FOR ^{3}H USING HUMAN TESTICULAR BIOPSY SAMPLES

Experiment No.	*Peak*	*Flo-One HP* (cpm)*	*Fraction collection (dpm)***	*Efficiency (%)****
1	1	160,413	422,000	38.0
	2	62,529	155,850	40.1
	3	78,570	177,720	44.2
	4	403,867	964,830	41.9
	5	213,108	528,710	40.3
	6	46,466	114,100	40.7
2	1	128,531	337,340	38.1
	2	47,526	110,660	43.1
	3	131,950	131,950	45.8
	4	217,108	493,200	44.0
	5	95,227	225,190	42.3
	6	25,796	57,460	44.9
3	1	146,537	386,070	37.8
	2	58,922	133,580	44.1
	3	93,825	230,600	40.7
	4	354,754	818,100	43.3
	5	168,019	367,300	45.7
	6	197,172	460,990	42.8

* Cpm calculated in scalar mode.
** Dpm corrected by liquid scintillation counter.
*** Mean = 42.24%; standard deviation = 2.81%; standard error = 0.57%.

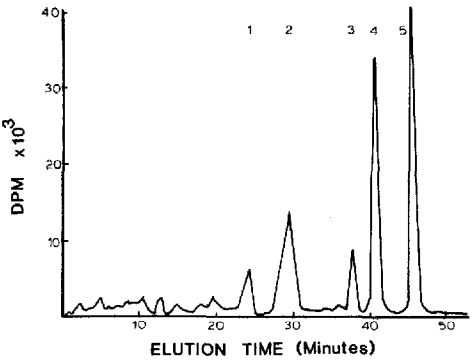

Fig. 3. Separation of steroid metabolites from the incubation of [^{3}H]pregnenolone with rat Leydig cell cultures. Quantitation by Flo-One HP radioactivity detector, using Flo-Scint II as scintillation fluid. Column and HPLC conditions as in Fig. 1. Elution positions of the five peaks 1–5 also identified by comparison with standard steroids as (1) androstenedione, (2) testosterone, (3) dihydrotestosterone, (4) progesterone and (5) pregnenolone. The apparent decrease in peak width for early eluting peak is due to the fact that some of the early peaks consist of two unresolved peaks.

three different human testicular biopsy samples, with the counting efficiency for ^{3}H shown in Table II. The mean efficiency was 42.2% with a small standard deviation (2.8%) and standard error (0.57%). The peak resolution was not substantially affected by the use of the stream splitter, as shown in Fig. 3, in which 50% was collected and counted and 50% was analyzed directly using the flow-through radioactivity detector.

These studies indicate that the flow-through radioactivity detector with the liquid mixing cell allows the rapid and quantitative analysis of radiolabeled compounds eluting from the HPLC column, but the reproducibility should be considered. A sample obtained from a metabolic study with a Leydig cell culture using [^{3}H]pregnenolone was used to study the reproducibility of the flow-through radioactivity detector. With the Flo-One HP detector, two methods were used for analysis of the data. The first method was the scalar mode, in which the counts per minute are

TABLE III

REPRODUCIBILITY OF FLOW-THROUGH RADIOACTIVITY DETECTOR —SCALER MODE

Peaks are from Fig. 3.

*Sample No.**	*Disintegrations per minute*				
	Peak 1	*Peak 2*	*Peak 3*	*Peak 4*	*Peak 5*
1	15,221	48,658	19,929	76,643	94,547
2	15,817	52,975	19,814	82,014	99,473
3	15,959	47,786	20,149	80,451	102,343
4	14,744	47,035	19,286	82,956	101,393
5	13,913	48,808	20,219	79,538	103,403
Mean	15,130	49,052	19,879	80,320	100,231
Standard deviation**	5.5%	4.7%	1.8%	3.0%	3.5%
Standard error**	2.5%	2.1%	0.8%	1.4%	1.6%

* Leydig cell culture sample on reversed-phase C_{18} (3 μm) column.

** Standard deviation and error of percent counting efficiency.

TABLE IV

REPRODUCIBILITY OF FLOW THROUGH RADIOACTIVITY DETECTOR–INTEGRATOR MODE

Peaks are from Fig. 3.

*Sample No.**	*Disingetrations per minute*				
	Peak 1	*Peak 2*	*Peak 3*	*Peak 4*	*Peak 5*
1	15,010	48,298	22,100	78,156	104,243
2	14,616	46,314	19,918	88,105	101,974
3	14,124	46,384	18,768	75,730	101,044
4	15,090	46,624	17,500	77,126	95,182
5	15,782	46,318	18,962	77,329	97,080
Mean	14,924	46,787	19,449	77,068	99,904
Standard deviation**	4.1%	1.8%	8.0%	3.7%	3.7%
Standard error**	1.8%	0.8%	3.9%	1.7%	1.7%

* Leydig cell sample on reversed-phase C_{18} (3 μm) column.
** Standard deviation and error of percent counting efficiency.

analyzed at pre-set time intervals (*e.g.*, 0.5 min). The second method was the integrate mode, in which each of the peaks of radioactivity was quantitated by integration of the number of disintegrations per minute or counts per minute in the entire peak with the total area and retention time being printed out. Both methods were compared. Table III shows the reproducibility of five consecutive runs in the scalar mode, with five peaks of radioactivity ranging in areas from 15,000 to 100,000 dpm in the sample. The standard deviation was 2–5% and the standard error of the mean 1–3%, which were well within the range obtained for five samples using the fraction collect method. Table IV indicates that even in the integrate mode of peak analysis the standard deviation and error were in about the same range as for the scalar mode. Finally, in five runs in the scalar mode and five runs in the integrate mode (Table V), the overall

TABLE V

REPRODUCIBILITY OF FLOW-THROUGH RADIOACTIVITY DETECTOR APPLIED TO STEROID SAMPLES

Leydig cell culture sample on reversed-phase C_{18} (3 μm) column.

Peak No.	*Mode of analysis*					
	*Scalar (n = 5)**		*Integrate (n = 5)**		*Scalar + integrate (n = 10)**	
	*S.D.**(%)*	*S.E.**(%)*	*S.D.**(%)*	*S.E.**(%)*	*S.D.**(%)*	*S.E.**(%)*
1	5.5	2.5	4.1	1.8	4.6	1.5
2	4.7	2.1	1.8	0.8	4.2	1.4
3	1.9	0.8	8.8	3.9	6.0	1.9
4	3.0	1.4	3.7	1.7	3.9	1.2
5	3.5	1.6	3.7	1.7	3.4	1.1

* n = Number of injections.
** Standard deviation; S.E. = standard error. Mean S.D. = 4.4% and mean S.E. = 1.4% for all 50 peaks resolved and counted in the Flo-One HP detector.

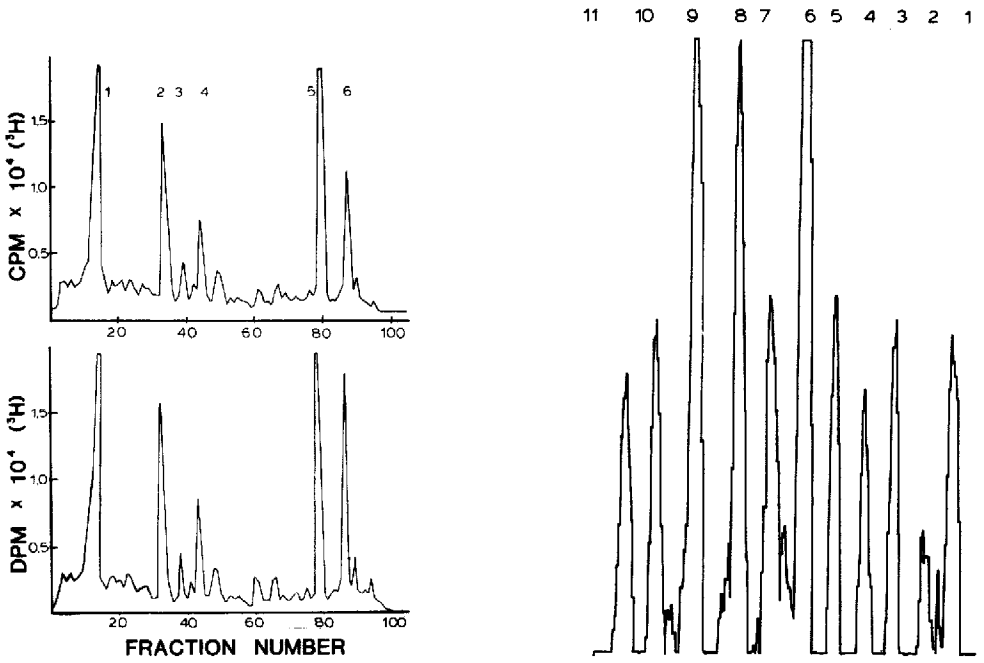

Fig. 4. Comparison of the separation and detection of radiolabeled metabolites of [^{3}H]pregnenolone from the *in vitro* incubation of monkey testis. The radiolabeled steroids were separated on a 3-μm C_{18} reversed-phase stainless-steel column (15 × 0.62 cm) at a flow-rate of 1.0 ml/min using methanol and water as described in Fig. 1 and ref. 6. After the HPLC separation, the eluent was split with the electronic variable ratio stream splitter with 50% proportioned to the fraction collector and 50% being analyzed directly by the Flo-One HP radioactivity detector. The upper panel shows the results obtained by the fraction collection method (0.5 min per fraction) corrected to the entire sample. The lower panel shows the direct analysis with the Flo-One HP radioactive detector with the counts per minute printed out every 0.5 min using an eluent to scintillator (Flo-Scint II) ratio of 1:3 (v/v). The numbers represent the elution positions (times) of standard steroids: (1) 19-hydroxytestosterone; (2) androstenedione; (3) estradiol; (4) testosterone; (5) progesterone; (6) pregnenolone (included as recovery steroid).

Fig. 5. Sensitivity of Flo-One HS flow-through radioactivity detector using the liquid mixing.cell (2500 μl) with a ratio of Flo-Scint II to effluent of 3:1 (v/v). The ratemeter output at the Flo-One HS (100 dpm full scale) was plotted by the Waters data module. Peaks 1, 3, 4, 5, 7, 10 and 11 contain 300 dpm; peak 2, 75 dpm; peak 6, 1200 dpm and peaks 8 and 9 contain 600 dpm of [^{3}H]-testosterone injected on a 3-μm C_{18} column, flow-rate is 1.5 ml/min.

standard deviation was 4.4% (range 3.4–6.0%) and standard error was 1.4% (range 1.1–1.9%). This demonstrates that the flow-through radioactivity detector gives reproducible, rapid and quantitative results further shown in Fig. 4. A study of the problem of contamination from one sample to the next showed that there was about 0.1% or less carry-over in the HPLC system and much less (0.05%) in the Flo-One HP radioactivity detector.

The final question to be addressed is the sensitivity of the flow-through radioactivity detector for low-energy β-emitters (^{3}H). For these studies a highly sensitive radioactivity detector (Flo-One HS) was employed. This detector was able to easily detect and quantitate peaks of 100 dpm or greater, as shown in Fig. 5, with a very low background. This sensitivity is accomplished by increasing the size of the

TABLE VI

REPRODUCIBILITY OF INJECTION OF [^{3}H]TESTOSTERONE SAMPLE (350 dpm) ONTO HPLC COLUMN AND QUANTITATION USING FLO-ONE HS DETECTOR

Injection No.	*Dpm/peak**
1	286
2	393
3	270
4	304
5	369
6	373
7	331
8	336

* Mean = 332 dpm; standard deviation = 43 dpm; standard error = 15 dpm; coefficient of variation = 13.21%; counting efficiency = 25% ^{3}H.

flow cell for the scintillation fluid–effluent mixture to 2.5 ml, using a precise HPLC pump for accurate control of the flow-rate of the scintillation fluid and employing a new data reduction software package.

The reproducibility of eight consecutive injections of the same sample (350 dpm total area) is shown in Table VI. Even at a sensitivity of 350 dpm the standard deviation is 43 dpm and the coefficient of variation is 13.2%. Considering this is a direct on-line analysis of the disintegrations per minute, this is excellent reproducibility at this level of sensitivity.

In conclusion, the flow-through radioactivity detector with the homogeneous counting cell employing a liquid scintillation fluid offers a highly sensitive, rapid, reproducible and quantitative method for analyzing either ^{14}C or ^{3}H peaks or peaks containing both ^{14}C or ^{3}H eluting from the HPLC column.

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