DETERMINATION OF SPECIFIC ACTIVITY OF TRITIUM LABELLED COMPOUNDS BY GAS-LIQUID RADIOCHROMATOGRAPHY AND LIQUID RADIOCHROMATOGRAPHY

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ABSTRACT

Methods for determining the specific activity of tritium-labelled compounds are reported. Separation and analysis are by gas-liquid radiochromatography and liquid radiochromatography. Mass of the compound is measured by electronic integration with a sensitivity in the nanomole range, and the radioactivity is determined by proportional flow counting or by liquid scintillation counting of collected fractions. Two methods for quench correction in the gas flow counter are given. Monitoring the presence of [^{3}H]iodobenzene in solution with a liquid flow scintillation system using glass scintillators is insensitive and impractical. The insensitivity may be caused by annihilation quenching by some of the analytes that annihilate β-particles before they impinge on scintillation solids.

INTRODUCTION

In isotopic labelling and tracer applications, one needs to know the specific activity of labelled compounds. When sufficient material is available, quantifying the mass by weighing, uv absorbance and other analytical techniques presents no difficulty. When no-carrier-added labelled compound is involved, the available amount of labelled material may not be sufficient for such methods of estimation, in which case one may assume that the specific activity of the product is

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identical with that of the radioactive starting material provided that contamination by non-radioactive precursors does not occur during synthesis. If such contamination cannot be excluded, it is necessary to measure the specific activity of the final labelled product by more sensitive means of quantification, such as receptor binding assay, polarography, etc. Fowler _et al._[1,2] determined the specific activity of ^{11}C-labelled radiopharmaceuticals by these techniques. Peng[3] reported the use of gas-liquid radiochromatography (GLRC) for assessing the specific activity of labelled products formed by plasma-induced incorporation of radiocarbon dioxide into organic compounds. The ability to determine the specific activity of individual components in a product mixture is also a valuable technique for kinetic studies. This report describes a method for measuring the specific activity of individual compound such as [^{3}H]iodobenzene, the presence of which depresses the detector response in the gas counter as well as in solution in a scintillation flow detector. It also sets the upper limits at which the specific activity of no-carrier-added labelled compounds can be determined by GLRC and by high performance liquid radiochromatography (HPLRC).

EXPERIMENTAL

Materials used in the experiments were either purchased from commercial sources or obtained by tritium labelling. [^{3}H]Toluene and [^{14}C]toluene were from New England Nuclear Incorporated (Boston, Massachusetts). Tritium-labelled iodobenzene was obtained from iodobenzene by reaction with specially prepared supported catalysts charged with tritium[4]. Some of these samples also contained radio-impurities formed by radiation-induced labelling or from reactions with unidentified reactive tritium species.

Analysis by GLRC was performed on a Hewlett-Packard 5880A Gas Chromatograph with a thermal conductivity detector (TCD) and a Level IV integrator terminal. Samples were analyzed isothermally or with programmed temperature on a stainless steel column, 3.05 m x 3.18 mm O.D., packed with 80-100 mesh Supelcoport coated with 10% Carbowax-20M or on a column, 1 m x 3.18 mm O.D., packed with 100-120 mesh

Porapak QS[5]. The oven temperature was programmed to begin at 70°C for 10 minutes, rising at a rate of 10°C per minute to a final temperature of 210°C for 10 to 20 minutes. In some runs, the initial temperature was 150°C. The low initial temperature was for separation of low-boiling radioactive impurities that were formed during the process of tritiation. Isothermal runs were carried out at an oven temperature of 120°C. Temperatures of other heated zones in the chromatograph were: injection port 230°C, detector 250°C, and auxiliary (sample lines) 230°C.

The thermal conductivity detector in the H-P 5880A Gas Chromatograph is a single filament detector and requires a high helium flow for purging; the overall flow rate for these studies was approximately 90 ml/min. According to manufacturer's specification,[6] the TCD has a sensitivity of 1 microvolt, corresponding to 1 cm in peak height at zero attenuation. Area integration is based on slicing and the size of the area is reported as area counts[7]. When calibrated against a standard compound, the area counts can be equated with the mass of the compound.

For radioactivity determination, the effluent of the chromatographic column was mixed with twice its volume of n-propane before passing through a proportional counter with a volume of 85 ml., heated to 250°C to prevent sample condensation. Radioactive peaks were recorded in a Tracor-Northern TN7200 Multichannel Analyzer operating in the multichannel scaling mode, and the integration of the peak activity was performed with the region-of-interest feature in the instrument. The counter had a voltage plateau in excess of 300 volts and an efficiency of 11 to 13% based on calibration with a standard of [^{3}H]toluene solution. A ^{60}Co source was also employed for monitoring the performance of the counter tube. Contamination inside the counter tube was removed by oxidation by passing air through it at 300°C overnight.

The high performance liquid radiochromatography (HPLRC) was carried out with a Beckman-Altex Model 330 Isocratic Liquid Chromatograph operating in the reverse-phase mode. For separation of a mixture of benzene and iodobenzene on an Ultrasphere ODS C_{18} column (250 mm x 4.6 mm I.D.), a solvent mixture of methanol and water

(70:30, v/v) was used. In this solvent mixture, the k' values for benzene and iodobenzene are 1.96 and 4.93, respectively. For radioactivity measurement the eluate was collected in 0.5 ml fractions with a Pharmacia Fraction Collector FRAC-100 into pico glass counting vials with an 8 ml volume; after addition of 4.0 ml of Insta-Gel scintillant (Packard, Warrenville, Illinois), the samples were counted in a Beckman LS 9000 Liquid Scintillation Counter with a 42% unquenched efficiency. Liquid radiochromatograms were obtained as nomographic plots.

RESULTS AND DISCUSSION

The area counts for benzene, cyclohexane, iodobenzene, and toluene differ from one another on a micromole basis (Table I). The area count is directly proportional to the mass. In the case of iodobenzene, a plot of area counts <u>versus</u> concentration is linear over a concentration range of four orders of magnitude (Figure 1). The high sensitivity of TCD permits a ready detection of sample mass in the nanomole range. This is the upper limit of the specific activity that no-carrier-added labelled analyte can be accurately determined by GLRC.

Table I. Response Factors for Different Compounds by Electronic Integration[a]

Compound	Response factor, area counts μM, x 10^5		
	GLRC[b]		HPLRC[c]
	Carbowax-20m[d]	Porapak QS[e]	Ultrasphere ODS-C_{18}[f]
Benzene	2.97 ± 0.05	5.31 ± 0.16	0.2 ± 0.003
Cyclohexane	4.12 ± 0.03	6.76 ± 0.24	---
Iodobenzene	5.18 ± 0.16	6.87 ± 0.52	1.83 ± 0.05
Toluene	4.01 ± 0.05	6.50 ± 0.19	0.13 ± 0.003

[a]See text

[b]GLRC = Gas-liquid radiochromatography

[c] HPLRC = High-pressure liquid chromatography

[d] A stainless steel column, 3 m x 3.2 mm dia. O.D., packed with 80-100 mesh 10% Carbowax-20M on Supelcoport.

[e] A stainless steel column, 1 m x 3.2 mm dia. O.D., packed with 100-120 mesh Porapak QS.

[f] An Ultrasphere ODS-C_{18} column 5 μM, 25 cm x 4.6 mm dia. O.D.

The presence of a certain amount of some organic compounds in gas counters causes quenching by terminating the counter discharge; the cause is due to the absence of secondary electrons[8]. Halogenated organic compounds are quenchers in this respect. For this reason, the method for determining specific activity by GLRC is only useful and valid when the counting loss caused by quenching in the gas phase is precisely known.

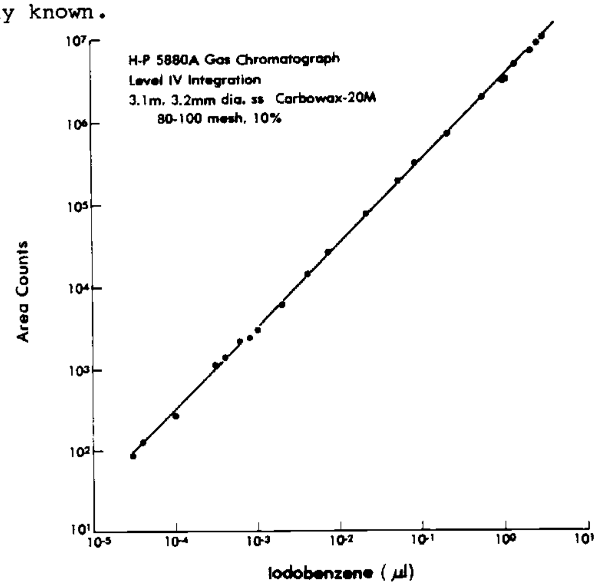

Figure 1. Area counts vs. concentration of iodobenzene

Figure 2 shows the quenching curves for ^{60}Co, ^{14}C, and ^{3}H, correlating the percent counting loss due to quenching with the concentration of iodobenzene. These curves were obtained under identical experimental conditions for analysis with the exception that the chromatographic columns were replaced with empty columns so that no separation of the injectate (2-5 μl) could occur. The quench curve for ^{60}Co was obtained by placing a ^{60}Co source directly over the flow counter to give a steady count rate of Compton electrons and gamma rays and then injecting different amounts of non-radioactive iodobenzene to cause a temporary decrease of the count rate. When the maximum decrease of the ^{60}Co count rate was plotted against the injected iodobenzene concentration, an S-shaped curve resulted as shown in Figure 2. Similar quench curves were obtained for ^{3}H and ^{14}C by injecting mixtures containing a given amount of [^{3}H]toluene or [^{14}C]toluene and increasing amounts of iodobenzene. Since the iodobenzene and the labelled toluene could not be separated by the empty column, their instantaneous passage through the proportional counter would be equivalent.

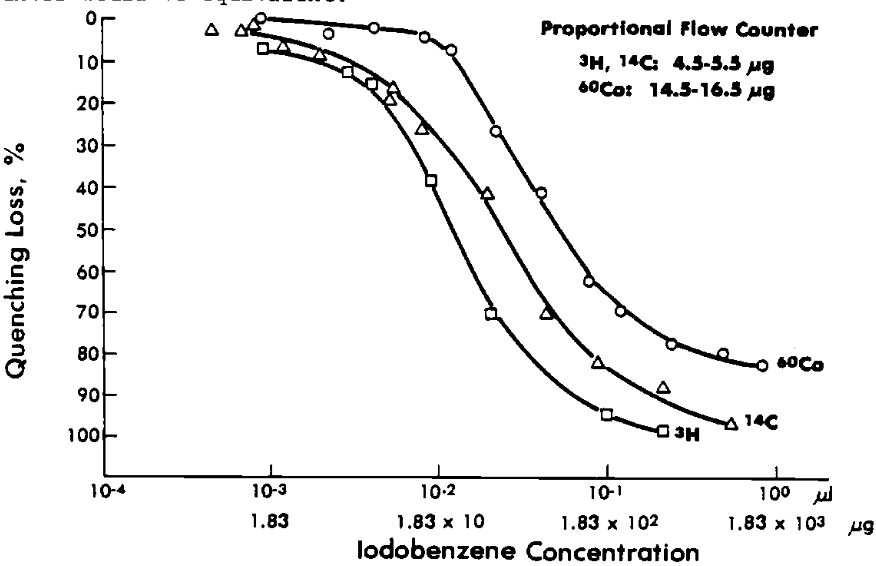

Figure 2. Quenching curves relating quenching loss to iodobenzene concentration for ^{60}Co, ^{14}C, and ^{3}H. See text.

The three quench curves possess similar characteristics in having distinct regions. In the initial region, below a certain amount of iodobenzene in the gas counter, the count loss by quenching is minimal; in the central region, the counting losss increases linearly with increase in iodobenzene concentration; and in the final region, the count loss reaches a maximum and shows only slight increase with further increase in iodobenzene concentration. The steep portion of the plot in the upper part of the central region can be extrapolated to zero quench loss to yield the concentration of iodobenzene 4.5 to 5.5 μl for ^{3}H and ^{14}C and 14.5 to 16.5 μl for ^{60}Co. That is, at or below these concentrations of iodobenzene, the count rates of these radionuclides would not be quenched. This is the basis of our method for correction of quenching in the gas counters.

Figure 3 shows a quench curve obtained by injection of mixtures of the same quantity of [^{3}H]iodobenzene (DPM determined by LSC: 185,937) and different amounts of non-radioactive iodobenzene. The response of the proportional counter in DPM of the sample is plotted against the area counts for the total amount of iodobenzene (radioactive plus non-radioactive) on a log-log paper to yield a straight line which can be extrapolated to a reference line of 5 μg iodobenzene concentration to give the DPM of the sample as 183,283. The discrepancy between the extrapolated value and the value determined by LSC is less than 1.5%. To correct for quench in a sample of iodobenzene of undetermined specific activity, the sample is first chromatographed, and the same quantity of radioactive sample is diluted with various amounts of inert compound and chromatographed. The area counts of these injectates and their corresponding radioactivity are plotted as shown in Fig. 3 and the plot is extrapolated to the 5 μg limit reference line to obtain sample radioactivity in the absence of quenching in the gas counter.

The above method for quench correction is based on the assumption that quenching is directly proportional to the amount of iodobenzene present in the gas counter; a rapid removal of its content is necessary to keep the quenching to a minimum and the elution time for the compound is not extended so as to obviate the effect of bolus injection. These conditions are only partially met in practice. The content of our gas counter due to the rapid flowrate, has a turnover time of about 20 seconds. The chromatographic peak for large quantities of iodobenzene is very wide and requires several minutes for complete elution. As a result, the radioactivity peak matches the width of the mass peak at the base but is truncated at the top.

Another method of quench correction was also investigated. It

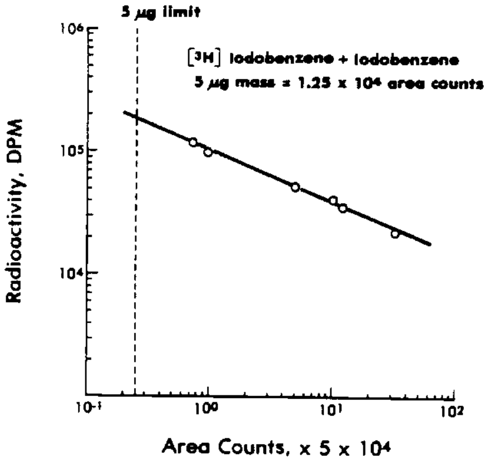

Figure 3. A self-quench curve for [^{3}H]iodobenzene. See text.

involves analyzing the radioactive sample repeatedly in different multiples of a unit quantity without dilution by the inert compound. The radioactivity observed for the injectate should be either identical with or less than the multiples of the radioactivity for that quantity. The decrease in count rate is dependent upon the quenching property of the compound. The count loss will be proportionately greater when the volume of the injectate is large than when it is small. If the apparent specific activity or the unit radioactivity is plotted against the concentration of the injectate on a semi-log paper, a linear relationship results. The relationship was first observed in liquid scintillation counting of some ^{35}S-labelled thioureas[9]. Extrapolation of the linear plot to zero concentration of the injectate will yield the specific activity of the compound in the absence of quenching in the gas counter. Table II shows the interrelationship of the volume of injectate, area couunts, unit multiples, CPM, and CPM per unit volume of injectate.

Figure 4 shows the plots of the observed unit radioactivity of sample vs. the injected volume of purified preparation of [^{3}H]iodobenzene (Samples 2a and 2a$_1$) (broken lines). The area counts in units of 50,000 were used for normalization to verify the injected volume (solid lines). The plots were extrapolated to intercept the

Table II. Data Computation for Quench Correction by Extrapolation Method in GLRC[a].

(A) Volume injected (1)	(B) Area Counts	(C) Unit Multiples[b]	(D) Activity Observed (CPM)	(E) Unit Activity[a] (D)/(C)
[^{3}H]Iodobenzene - Sample 2a				
0				(5,372)[d]
2.0	50,731	1.015(2.03)[e]	10,693	5,267
4.0	98,127	1.96 (3.92)	20,173	5,146
6.0	154,544	3.09 (6.18)	31,180	5,045
[^{3}H]Iodobenzene - Sample 2a$_1$				
0				(2,851)[d]
1.0	230,793	4.62(0.924)[e]	2,385	2,581
2.0	506,580	10.13(2.026)	3,576	1,765
4.0	986,465	19.73(3.946)	4,456	1,130
6.0	1,492,780	29.86(5.972)	4,683	784

[a]See text and Figure 4.

[b]A value of 50,000 area counts is selected to represent a unit, and the unit multiple is obtained by dividing the recorded area counts (B) by this value.

[c]The unit activity is the quotient of the activity observed (D) by the unit multiple (C). It represents the apparent activity of the analyte.

[d]Extrapolated value to zero quench obtained by the least square method of curve fitting.

[e]The figure in parenthesis is the value of unit multiples divided by 5 to yield the corrected volume for the plot.

ordinate to give the unit activity of these samples in the absence of quenching. The unquenched unit activities for Samples 2a and 2a$_1$ were 47,531 and 25,230 DPM/50,000 area counts, respectively. Sample 2a$_1$ was the dilution of Sample 2a by an equal volume of an n-hexane solution containing 10% of inert iodobenzene. The discrepancy between the extrapolated value and the true value is about 6%, indicating the usefulness of the method for correcting quenching in the gas phase. Some of the discrepancy may also be attributed to difficulties encountered in manipulating minute volumes of highly radioactive samples and short-term drifting of the radioactivity monitoring instrument.

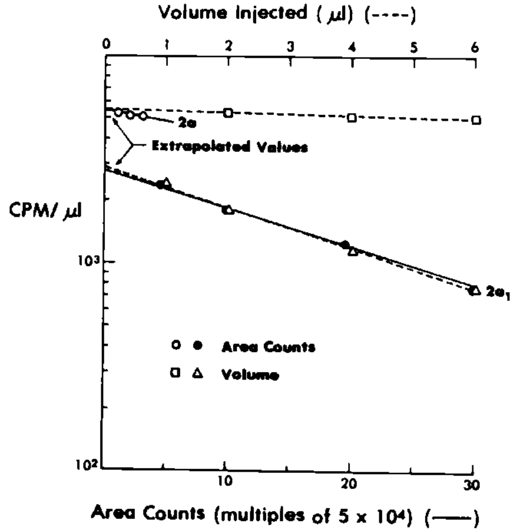

Figure 4. Extrapolation plots of unit multiples of [^{3}H]iodobenzene <u>vs</u>. activity. See text.

<u>Liquid chromatography</u>. Radioactive samples may also be analyzed by high performance liquid radiochromatography (HPLRC). The radioactivity of the eluate is usually determined using scintillation flow cells packed with plastic or glass scintillation beads or by mixing the eluate with liquid scintillant in a flow system. It may also be measured on fractions collected in counting vials followed by addition of liquid scintillator and counting. Quenching may be readily corrected in batch counting but would be difficult to correct in a flow system.

In our study of determining the specific activity of ^{3}H-labelled compound by HPLRC, we relied on the collection of the eluate for radioactivity measurement by liquid scintillation counting. Assay of [^{3}H]iodobenzene of high specific activity in multiples of a quantity in a flow system with cells packed with glass scintillation beads has not yet yielded the anticipated response. The flow detector showed some response when the injected volume is small but became insensitive towards large injected volumes of [^{3}H]iodobenzene; the flow detector exhibited negligible activity while the collected eluates were found by LSC to contain all the injected activities. The low efficiency for ^{3}H detection of this flow system has made it unsuitable for use for monitoring low-level activities in column effluents. The inability to respond quantitatively to the activity present in the sample may be attributed to the presence of an electron scavenger in solution which reduces the number of electrons impinging on the surfaces of the glass scintillator and results in less scintillation produced for the same amount of the sample that contains no quencher in solution.

This reduced response is probably caused by annihilation quenching[10]. Glass scintillators respond to tritium activity because β-particles from tritium decay impinge upon the surface and interact with the scintillator. Once the interaction is initiated, the presence of quencher in the solution has no effect on the energy transfer of the scintillation process; chemical or impurity quenching as we know it in solution does not occur in solid scintillators. But if the solution contains electron scavengers such as iodobenzene, their presence will exert an annihilation action on electrons in solution, resulting in the reduction of the number of electrons impinging upon the surface of the glass scintillator, and causing a disproportionately small detector response for the radioactivity present in solution. This phenomenon of annihilation quenching in flow counting has not been previously reported.

The area counts for the mass peak in HPLRC are based on the sensitivity of the uv detector at 254 nm. On a micromole basis, the uv detector for liquid chromatography is about 3 to 20 times less sensitive than the TCD in gas chromatography, as seen from the data given in Table I. Figure 5 gives the visual comparison of the nomograph representing the activity of [^{3}H]iodobenzene analyzed by HPLRC and the recorder tracing of the same compound by GLRC.

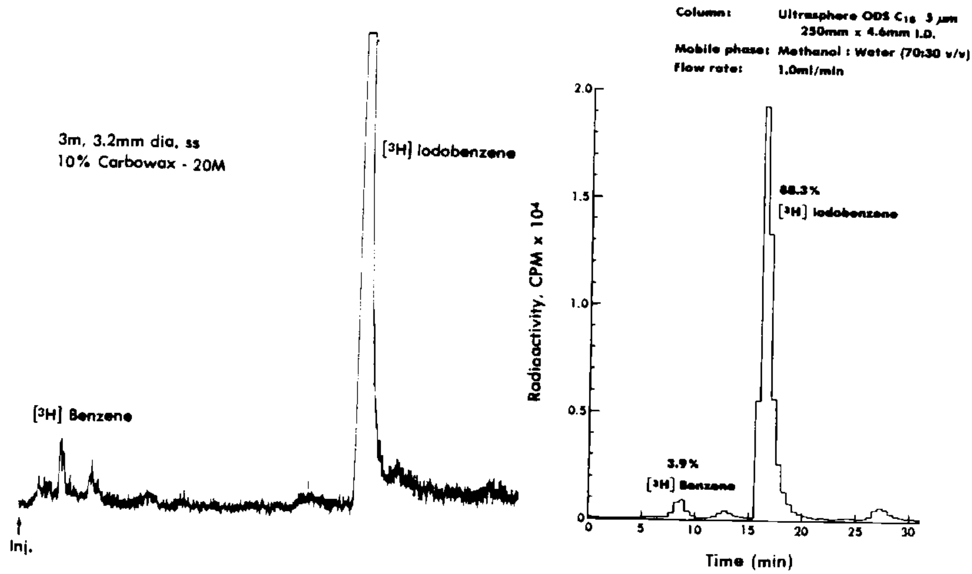

Figure 5. A comparison of the activity nomograph of [^{3}GH]iodobenzene by HPLRC with the recorder tracing of the same compound by LGRC. The % recovery of [^{3}H]iodobenzene by the two methods of separation and analysis is approximately identical.

CONCLUSION

Both GLRC and HPLRC, when properly equipped for area and radioactivity measurement, are invaluable for determining the specific activity of ^{3}H-labelled compounds. Some of the ^{3}H-labelled compounds are electron scavengers; their presence in the gas counter causes quenching which must be corrected if valid activity measurement is to be made. Quench correction may be carried out with the extrapolation method, based on the data of apparent specific activities (or unit radioactivities) observed at different sample sizes. The precision of the method is improved when minute amounts of highly radioactive materials are precisely handled. Commercial liquid scintillation flow monitoring systems using glass scintillator beads have low counting efficiency for ^{3}H; its efficiency is adversely affected by annihilation quenching. Our study indicates that annihilation quenching in flow counting may also be determined by measuring the radioactivities of the analyte at different concentrations; the reduced response for multiples as compared to the response for unit concentration of an analyte indicates annihilation quenching.

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