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Quantitative comparisons of reaction products using liquid chromatography with dual-label radioactivity measurements

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ABSTRACT

Dual-label methods for radiometric measurements with liquid chromatography are evaluated. The procedures show marked improvements in data quality for comparisons of product formations and relative product abundances in multiple-pathway reaction systems. The results from these procedures are shown to be relatively unaffected by large variations in uncertainties in pretreatment steps and volume measurements. Moreover, these methods yield much better quantitative results than do commonly used corresponding conventional comparisons.

These quantitative comparisons use reference substances generated by a reference reaction system as internal standards. A suite of reaction products is compared via the internal standards for components common to both reference and sample reaction systems. The dual-label methods are especially suitable for toxicologic metabolism comparisons, but could easily be adapted to other reaction systems such as syntheses or degradations. Moreover, the dual-label procedures should be amenable to non-radioactive measurements with isotope-selective methods such as mass fragmentometry, emission spectrometry or resonance methods.

INTRODUCTION

Radioactivity measurements for chromatography are especially helpful in experiments involving multiple reaction pathways, partly because radiochemical detection can provide good selectivity and high sensitivity. Resulting low limits of detection and freedom from many interferences can make radiometric procedures powerful quantitative methods. Recent work with chromatographic applications of radiometric detection has been reviewed [1] including commercial instruments available for flow-through measurements of radioactive eluates.

Several factors plague radiometric procedures for reaction product evaluations and can cause large uncertainties in resulting data and comparisons [2–4]. Variations in extraction efficiencies and variable losses during volume reductions can be partially compensated by traditional internal-standard techniques using differently labeled compounds which are otherwise identical to the target eluates. However, those procedures require both availability and careful characterization of appropriate reference materials for *every* measured component. Unfortunately, for complex systems such as metabolism studies, several reaction product analytes may be measured for each sample, which exacerbates difficulties associated with use of internal-standard meth-

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ods; moreover, identities of the products are not always known, which precludes use of conventional internal- or external-standard methods.

In previous work [2–4], compounds labeled with two different radioactive isotopes were used with high-performance liquid chromatography (HPLC) for special dual-label procedures which mimic internal-standard methods. In those studies biologically generated radiolabeled reference solutions were used and their components separated along with differently labeled respective coeluting metabolites from samples from biological experiments.

Two methods were described, each exploiting radioactivity detection of two labeled forms of each individual reaction product. One method employs a homogeneous reference solution of radiolabeled metabolites as a mixed internal-standard reference solution [2,4]. A known amount of the reference solution is added to each differently labeled experimental sample before sample preparation, but after biological reactions have taken place. This procedure may be used to quantitatively compare metabolism profiles and to test for differences between control *vs.* test groups in metabolic efficacy.

The second method uses a combined solution of differently labeled forms of the same compound with concurrent exposure of the two forms into a biological system via addition of an aliquant of the mixed dosage solution [2,3]. They are thereby concurrently metabolized under identical conditions. The respective metabolites then serve as mutual internal standards for quantitative comparisons to evalute: (a) effects of impurities on metabolism experiments, (b) effects of isotope exchange upon experiments and (c) effects of kinetic differences between the forms which may be caused by their different respective masses.

Those dual-label methods allow for compensation for variations in extraction efficiencies, variable losses during concentration of extracts, imprecisions of volume measurements and uncertainties in specific activities of dosage compounds and metabolism products. Moreover, the procedures greatly obviate difficulties caused by unavailability of pure reference compounds.

Improvements from the dual-label methods upon quantitative biological experiments have been estimated for a few selected circumstances [2], but no detailed evaluations of data-quality enhancements offered by the dual-label techniques had been done. In this study, detailed evaluations of improvements provided by use of the dual-label procedures are reported.

THEORY

A main advantage of the dual-label procedures discussed above is that several special ratios yield well-defined, theoretically predictable results which may be tested statistically [2,3]. Two of those special ratios are evaluated in this work.

Dual-label reaction-product determinations

In the absence of pure standards, a fixed known volume, V_a, of a homogeneous internal-standard solution generated by a reference reaction system which contains several radioactive reference compounds can be used in place of a conventional standard solution made by mixing known quantities of pure radiolabeled substances [2,3]. One may add these *X*-labeled reference compounds to subsamples of mixtures of

Y-labeled compounds of unknown concentrations which have been generated from reactions being investigated. By judicious selection, some of the *X*-labeled components will be chemically identical to the *Y*-labeled components, except for their respective radioactivities. Hence, pretreatments of the mixtures can yield equivalent extraction/concentration/dilution efficiences, E_i, for the two extractable forms of each common component. Thus, if V_t is the volume of the prepared subsample, and V_{ss} is the volume of the prepared subsample used for chromatographic separation, then $E_{Y,i} = A_{X,i}V_tA_{s,X,i}^{-1}V_{ss}^{-1} = E_{X,i}$, where $A_{X,i}$ and $A_{s,X,i}$ are corrected measured radioactivities for the *X*-labeled component *i* in the volumes V_{ss} and V_a, respectively. This equivalence is a reasonable assumption when isotope exchange is negligible, and the two forms are chemically alike and are not entrapped or bound in tissue or precipitates [2,3].

This method is similar to use of several radiolabeled internal standards using conventional radiochemical calculations and counting procedures. Consequently, the mass of each *Y*-labeled component from the sample can be determined by using that component's subsample activity data from the dual-label chromatogram, $A_{X,i}$ and $A_{Y,i}$, the total *X*-label activity in volume V_a for the reference solution, $A_{s,X,i}$, and the analyte specific activity, S_Y; $M_{Y,i} = A_{Y,i}A_{s,X,i}S_Y^{-1}$. This approach can be useful but is subject to several uncertainties which are avoided by use of the *R*- and *U*-ratio methods discussed below.

Single-component comparisons using the R ratio

In many experiments the absolute masses of analytes are often less important than their relative concentrations between experiments, *e.g.* in comparisons of metabolism in control *vs.* test organisms [5,6]. Such comparisons are suitable for use of multiple internal standards generated by a reference reaction system and the dual-label procedures described herein.

One may expose two sets of reactions, 1 *vs.* 2, to the same homogeneous dosage of *Y*-labeled compound, add volume V_a of *X*-labeled reference solution to each resulting sample, and then pretreat and separate each by liquid chromatography. Of course, $S_{Y,1} = S_{Y,2}$, as the dosage specific activities must be identical, and if $V_{a,1} = V_{a,2}$, then $A_{s,X,1} = A_{s,X,2}$. Moreover, if $V_{t,1} = V_{t,2}$ and $V_{ss,1} = V_{ss,2}$ by design,

$$R_{12} = (M_1/M_2) = (A_{Y,1}A_{X,2})(A_{Y,2}A_{X,1})^{-1} \tag{1}$$

for the component of interest, and this *R* ratio is calculated from counting data only.

If reaction efficacy is hypothesized to be not different between two groups, *e.g.*, control *vs.* test groups, then $R_{12} = 1$ if this null hypothesis is valid and R_{12} can be tested statistically to ascertain if it is different than unity for the specified component of interest, *e.g.*, if the compound reacts differently in the two systems.

Multiple-component comparisons using the U ratio

If the procedure used for the *R* ratio above is extended to several reaction products, then the resulting multiparametric method can be used to characterize several reaction pathways. Thus, relative magnitudes of several intrasample parameters may be tested and yield results which are more important than their absolute magnitudes or individual single-component comparisons between groups. For this,

an extension of the dual-label coelution method can be formulated for groups 1 *vs.* 2 and components *i vs. j* such that

$$U_{12} = (R_{12,i}/R_{12,j}) = A_{Y,i,1}A_{X,j,1}A_{Y,j,2}A_{X,i,2}A^{-1}_{Y,j,1}A^{-1}_{X,i,1}A^{-1}_{Y,i,2}A^{-1}_{X,j,2} \quad (2)$$

where X and Y represent the measured radioactive forms, subscripts i and j indicate the two components of interest, the subscripts 1 and 2 indicate samples from which the components were isolated and A indicates corrected measured radioactivities. If that reaction product profile for both groups is the same, then $U_{12} = 1$. This U ratio may be tested statistically, and the null hypothesis of identical relative reaction rates for those components may be assumed unless U_{12} is shown to be significantly different than unity.

Of course, by comparing several components, *i, j, k, l...*, one may evaluate reaction product profiles representing several modes *e.g.*, several metabolism pathways.

EXPERIMENTAL

Reagents

All organic solvents used were Mallinckrodt ChomAR. Radiolabeled compounds were purchased from Amersham. Water used was distilled in all-glass apparatus and other chemicals were reagent-grade quality.

Apparatus

HPLC. An Altex HPLC system with two Model 110 pumps, a Model 420 control module and a Beckman Model 171 dual-channel flow-through radioactivity counter interfaced to an Equity I+ computer and an Epson Model 810 printer was used. Separations were done on an Altex 25 cm × 4 mm I.D. stainless-steel column packed with 5-μm octadecylsilyl reversed-phase column material. Eluted fractions were collected in scintillation vials by a Pharmacia FRAC-100 fraction collector after automatic mixing with Beckman Ready-Flow II scintillation cocktail in the Model 171. Collected fractions were counted on a Beckman Model 5000 liquid scintillation counter and corrected for energy overlaps according to conventional procedures. Labware used was treated with dimethyldichlorosilane in hexane, and washed with water and acetone prior to use.

Computer simulations. An Epson Equity II+ computer with EGA graphics, a 20-Mbyte hard disk, an Intel 80287 supplemental mathematics coprocessor and an Epson 286e printer were used for mathematical modeling of dual-label chromatography separations and measurements. Computer programs were written in Microsoft's Quickbasic 4.0 (listings available from author upon request).

Chromatograms from the simulations were generated for specified enzymatic reaction rates of aryl hydrocarbon hydroxylases (AHHs), using relative metabolite concentrations from others' data compilations [7]. For rats metabolizing benzo[*a*]pyrene (B*a*P) relative concentrations in Table I were used, with other minor metabolic products ignored.

Efficiencies of component recoveries from extractions, reduction of extract volumes and dilutions to appropriate volume were taken from other work using reason-

TABLE I

METABOLITE RETENTIONS, PERCENTAGE OF METABOLITES AND UNCERTAINTIES DUE TO PRETREATMENTS

Metabolite	Retention time (min)	Met (%)[a]	E/C/D Eff[b] ± S.D. (%; n = 1,2 or 3)
9,10-B*a*P-dihydrodiol	16	8	0.4 ± 0.05 n
7,8-B*a*P-dihydrodiol	24	6	0.4 ± 0.05 n
1,6-B*a*P-dione	31	7	0.5 ± 0.05 n
3,6-B*a*P-dione	34	8	0.5 ± 0.10 n
6,12-B*a*P-dione	38	7	0.8 ± 0.10 n
9-OH-B*a*P	45	11	0.8 ± 0.10 n
3-OH-B*a*P	54	40	0.9 ± 0.05 n
B*a*P	65		0.9 ± 0.05 n

[a] Percentage of metabolites; assumed same for both reference metabolism and sample metabolism.
[b] Extraction/concentration/dilution efficiency for specified metabolite.

able standard deviations [2,7] (see Table I). No differences in these efficiencies were assumed for the differently labeled forms, consistent with their nearly identical chemical properties [2,3]. Random variations in these efficiencies were generated by an accepted procedure [8]. Variations in volume measurements for the diluted extract, the added reference solution and the subsample taken for HPLC were generated using reasonable standard deviations for volume measurements [9] and random Gaussian deviations [8].

Masses for each respective metabolite in subsamples taken for HPLC were calculated from assumed but varied AHH activities in the respective reaction systems, uncertainties in pretreatment recoveries, uncertainties in volumes and the volumes used. Mass *vs.* time relations for components separated by HPLC were calculated from respective injected masses, assumed HPLC peak widths at half-maximum of 1.0 min and presumed Gaussian peak shapes. The activities of the two labeled forms eluting from the column over all 1-s intervals throughout the separation duration were calculated. These interval activities were then summed into consecutive intervals of 6-, 15-, 30-, 60-, 90- or 120-s durations to simulate contents of collected fractions of corresponding times (see Fig. 1). Radioactivities were assumed to be ^{3}H and ^{14}C for these calculations. The measured activity for each fraction was calculated from the contained masses, specific activities, appropriate counting efficiencies for the relative concentrations of solvents and mixed scintillation cocktail, and imposed random variations in counting according to accepted procedures and appropriate compensation for energy overlaps [8,10].

Histograms simulating counted elution fractions were generated and total measured activities for each respective metabolite and labeled form were calculated by conventional methods [10]. Those data were saved for later computations and statistical analyses.

Fifteen replicates were done for each variation in combinations of: (a) four values for AHH reaction rates (10^{0}, 10^{-1}, 10^{-2} and 10^{-3} I.U./dl) (I.U. = 10^{-6} mol/min) for the reference solution, *i.e.*, yielding reference metabolite radioactivities; (b) 20 values for AHH reaction rates for the sample solution (10^{-3}–$2 \cdot 10^{-7}$ I.U./dl),

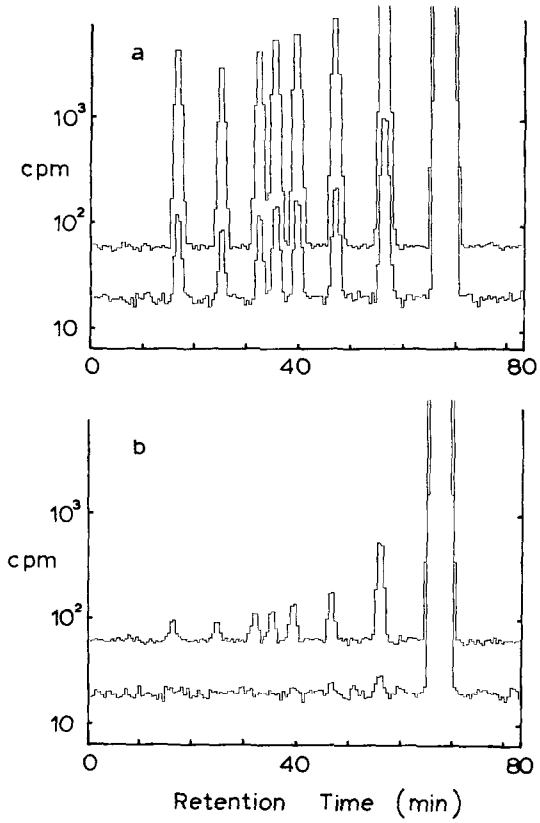

Fig. 1. Histograms of measured activity *vs.* time for 30 s eluted fractions. Upper chromatograms with approximately 49 cpm background are for ^{3}H from reference-solution eluates and lower chromatograms with approximately 16 cpm background represent ^{14}C components from samples. AHH enzymatic rates for the reference and sample incubations: (a) 10^{-1} I.U./dl for [^{3}H]B*a*P and 10^{-5} I.U./dl for [^{14}C]B*a*P, and (b) 10^{-3} I.U./dl for [^{3}H]B*a*P and 10^{-7} I.U./dl for [^{14}C]B*a*P.

i.e., yielding sample metabolite radioactivities; (c) three values for uncertainties in extraction/concentration/dilution efficiencies (1, 2 and 3 × S.D.; see Table 1 for values) and (d) six values for fraction-collection duration (6, 15, 30, 60, 90 and 120 s). Other values were held constant: (a) 10-min counting times were used; (b) 60-min enzymatic incubations were assumed for both reference and sample solutions; (c) specific activities of 500 and 325 mCi/mmol for ^{3}H-labeled and ^{14}C-labeled B*a*P, respectively; (d) 0.01 mol [^{3}H]B*a*P and 0.0001 mol [^{14}C]B*a*P added to reference and sample incubation solutions, respectively; (e) volumes of 25 ml for the total reference solution, 0.100 ml for reference solution added to sample prior to pretreatment, 100 ml for the sample incubation volume, 1.00 ml for the combined ^{3}H and ^{14}C mixture's resulting concentrate and 0.100 ml used for separation by HPLC, and (f) background count rates of 49 cpm for ^{3}H and 16 cpm for ^{14}C.

Measured radioactivities for each labeled form of each metabolite were saved and those data later were used to calculate *R* ratios within each replicate set for every metabolite-label pair and calculation of *U* ratios for all metabolites within each repli-

cate set relative to their respective 3-OH-B*a*P activities. Means (x) and standard deviations (S.D.) were computed and data quality was estimated as x/S.D. for each set of replicates. Confidence ranges ($x \pm$ S.D.) were calculated for R- and U-ratio results for the various replicate sets and plotted *vs.* AHH activities for sample solutions done under otherwise identical conditions.

Test organisms. Microsomes were prepared from tissue or isolated cells via homogenization in 1% KCl and centrifugation at 60 000 *g* for 60 min of the 20-min 20 000 *g* supernatant. Livers were excised from Sprague-Dawley rats (approximately 200 g) and treated immediately.

Procedures. Microsomes were incubated with reduced nicotinamide–adenine dinucleotide phosphate (NADPH), glucose-6-phosphate and glucose-6-phosphate dehydrogenase in oxygen-saturated 0.1 *M* PO_4^{3-} buffer at pH 7.5 with nicotinamide and B*a*P dosage compound at 37.5°C for rat-liver microsomes. After 2-h incubations, the metabolism was halted by additions of acetone. The incubates were then saturated with $NaCl_{(s)}$ and extracted four times with ethyl acetate. Descriptions of reagent volumes and concentrations may be found elsewhere [2,3]. The extracts were evaporated to dryness at 25°C under a stream of nitrogen and the residue partially dissolved into 1.0 ml of methanol. Aliquants (50 μl) of the methanolic concentrate were then separated by HPLC with the effluent being mixed with scintillation cocktail just prior to flow-through counting. The outflow from the Model 171 counter was collected as 1-min fractions in glass scintillation vials and each was counted for 20 min each on a multichannel liquid scintillation counter.

Data were plotted as histograms and net activities calculated for the eluted components. Eluate activities were then used for computations using eqns. 1 and 2, and for statistical calculations.

RESULTS AND DISCUSSION

Dual-label chromatograms from simulations were developed and histograms representing collected fraction radioactivities *vs.* time for those dual-label chromatograms (Fig. 1) were like results expected from corresponding biological experiments [2,7]. The histograms spanned a wide range of variations including those for which all metabolite-peaks' radioactivities were easily calculated (Fig. 1a) and others for which many eluates were below the limits of reliable measurement (Fig. 1b), with some circumstances allowing easy measurement of one labeled form but difficult to unfeasible measurements for the other.

Precisions were generally better for individual conventional calculations involving simple ratios for ^{14}C-labeled components between chromatograms, as compared to corresponding R- or U-ratio comparisons. This is consistent with the increased imprecision expected due to inclusion of additional radioactivity data in the calculations, *e.g.*, (A_{c1}/A_{c2}) has less uncertainty than $(A_{c1}/A_{H1}) / (A_{c2}/A_{H2})$. However, these simple one-label ratios are much less useful (see Fig. 2) than the corresponding R and U ratios as they yield no theoretically valid result unless volume and efficiency uncertainties are eliminated experimentally, which is typically unfeasible.

R and U ratios, as predicted by theory, yield unity for metabolism via the same pathways and metabolic systems (Figs. 2 and 3), even if experimental variations fluctuate dramatically. This powerful predictive factor was confirmed in all our theo-

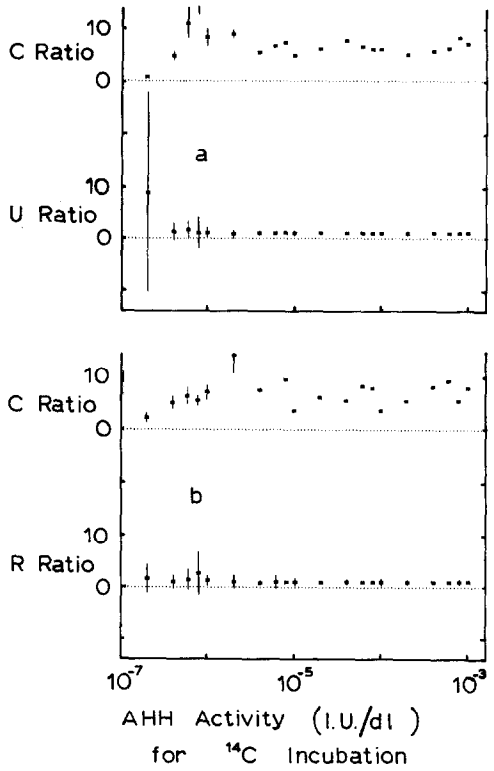

Fig. 2. Sample enzymatic rate (I.U./dl) *vs.* *U* ratios or corresponding ratios using only the C-label data: *U* ratio lower in each. These figures show results for two levels of extraction/concentration/dilution uncertainties: (a) ± 10% (R.S.D.); (b) ± 30% (R.S.D.). These data were attained using a reference solution for which AHH activity was 0.1 I.U./dl during incubation, *R* ratios for 6,12-B*a*P-dione and via *U* ratio comparisons between 6,12-B*a*P-dione and 3-OH-B*a*P.

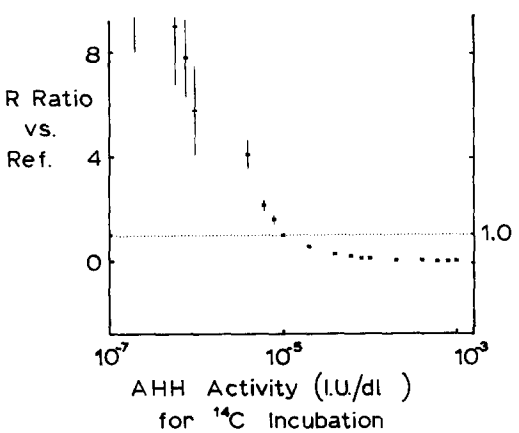

Fig. 3. *R* ratio *vs.* sample enzymatic rate (I.U./dl) for comparisons to metabolites from incubations with 10^{-5} I.U./dl AHHs in referenced sample incubation (Ref.) and 0.01 I.U./dl in the reference ^{3}H incubation. These data are for comparisons of 9,10-B*a*P-dihydrodiol between groups.

retical results, with typical standard deviations within replicate sets of only about ± 2–5% (R.S.D.), *e.g.*, 1.00 ± 0.03 with $n = 15$. Means of different replicate sets for U ratios varied little for similar reaction conditions and sample AHH values above 10^{-6} I.U./dl, with variations approximating 3–8% (R.S.D.), *e.g.*, 1.00 ± 0.05. R ratios showed somewhat better predictability than the U ratios, with values for $R = 1$ varying with about 3–5% (R.S.D.). Consequently, ratios for circumstances for which $R \neq 1$ or $U \neq 1$ are easily distinguished from those for which $R = 1$ or $U = 1$. Corresponding conventional one-label ratios varied greatly despite use of many replicates, typically with means of different replicate sets varying about ± 50–100% (R.S.D.), *e.g.*, 2.5 ± 2.0 (Fig. 2). This imprecision is due primarily to their lack of compensation for experimental variations. The advantages of the highly predictable, low-uncertainty U and R ratios are very important in regard to statistical hypothesis testing.

The magnitudes of measured radioactivities for reference-solution metabolites affects data quality for the corresponding R and U ratios. As expected, higher reference-component activities reduce the relative uncertainties in total-peak-activity calculations and thereby improve R- and U-ratio results. Of course, sufficient activity is necessary to ensure reasonable data quality (Fig. 1a *vs.* Fig. 1b), but very high activities provide only marginal improvements after reasonably good data quality is achieved (Fig. 4).

Interestingly, data quality for fairly long fraction-collection durations, *e.g.*, 60 s, was often better than that for shorter durations (6, 15 and 30 s) and was usually somewhat superior to that achieved for longer durations (90 and 120 s) (see Fig. 5). This observation is consistent with reduced ability to distinguish peaks when fewer data, *i.e.*, fractions, are used such as herein where the peak widths at half maximum are 1 min; that allows only several fractions to be used to characterize each peak. Conversely, using too many fractions detracts from the integrating ability of longer fractions, and can result in worse precision resulting from the diminished total counts for each fraction.

As extraction/concentration/dilution uncertainties increase greatly, corresponding R and U ratios become a little less precise. Moreover, the predictability, *i.e.*, the uncertainty for unity in the null hypothesis, changes only slightly due to those somewhat larger standard deviations in the R or U ratios (see Fig. 2). Thus, use of these ratios substantially reduces difficulties encountered due to large variations in recovery efficiencies and other pretreatment variables.

Increased numbers of replicates enhance data quality for the R and U ratios (see Fig. 6). As expected, improvements are significant for low numbers of replications, *e.g.*, 2 *vs.* 3, but the added relative advantage for high numbers is only slight. Thus, one may select a number of replicates which allows for appropriate statistical comparisons, *e.g.*, 3–5, and save the expense of using many repetitions of the corresponding experiments; when using conventional single-label comparisons, even very high numbers of replications do not result in the excellent predictability offered by only a few replicates with the R or U ratios.

Use of the dual-label procedures discussed above has the potential for dramatically improving statistical comparisons between reaction product profiles in experiments. This is mainly due to the theoretically valid predictability for the R and U ratios, *i.e.*, $R = 1$ or $U = 1$ for the null hypothesis. Also, because the added reference

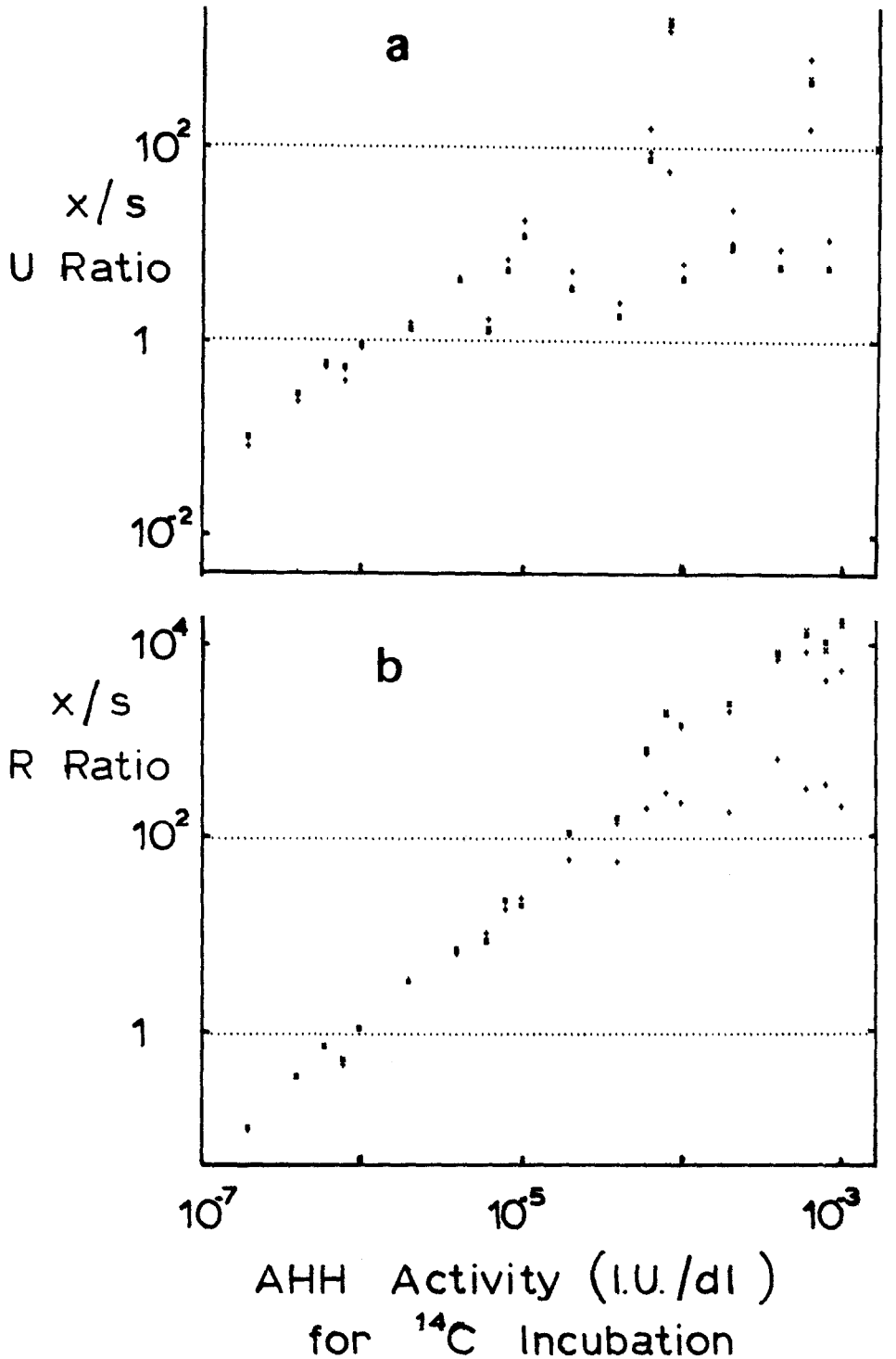

Fig. 4. (x/S.D.) for 3,6-B*a*P-dione with four AHH enzymatic rates in reference ^3H incubation, with 10% uncertainty in extraction/concentration/dilution efficiency; (a) for U ratios compared to 3-OH-B*a*P *vs.* sample AHH rates, and (b) for R ratios *vs.* sample AHH rates. ■ = 10^0 I.U./dl; × = 10^{-1} I.U./dl; + = 10^{-2} I.U./dl; ◇ = 10^{-3} I.U./dl. s = Standard deviation.

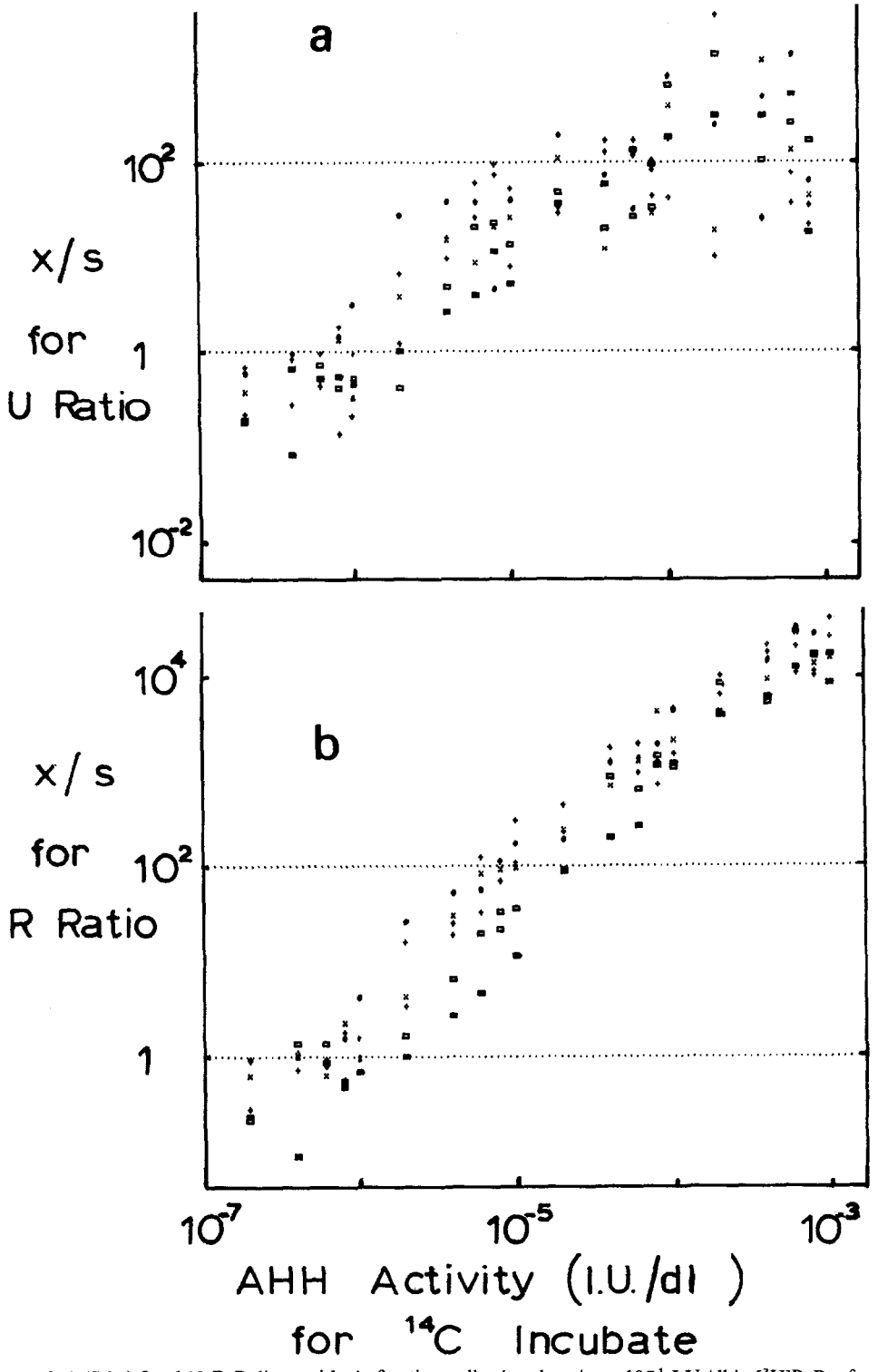

Fig. 5. (x/S.D.) for 6,12-B*a*P-dione with six fraction-collection durations, 10^{-1} I.U./dl in [^{3}H]B*a*P reference incubation and 10% extraction/concentration/dilution uncertainty. (a) for U ratios compared to 3-OH-B*a*P *vs.* AHH rates and (b) for R ratios *vs.* sample AHH rates. ● = 6 s; □ = 15 s; ◆ = 30 s; x = 60 s; + = 90 s; ■ = 120 s. s (In figure) = standard deviation.

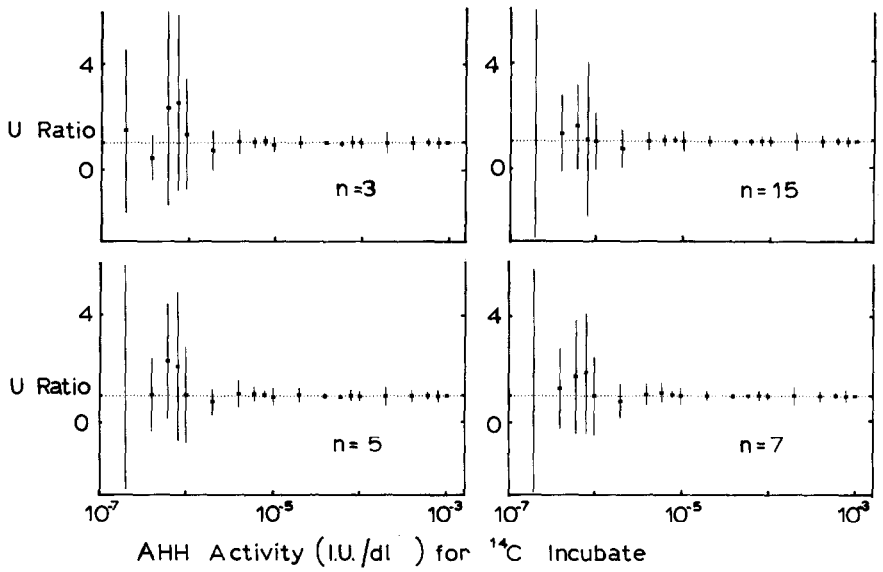

Fig. 6. *U* ratios *vs.* AHH rates (I.U./dl) for four variations in the number of replicates used for comparisons; these results are for 6,12-B*a*P-dione, with 10^{-1} I.U./dl AHH rate in the [^{3}H]B*a*P reference incubation and 10% uncertainty in extraction/concentration/dilution efficiency.

components are properly used as internal standards, the contributions due to uncertainties in recoveries, volume measurements, etc. are reduced appreciably, resulting in small standard deviations for corresponding *R* and *U* ratios. Moreover, because of the mathematical form of these ratios, needs for carefully defining specific activities and some other variables are obviated.

Results of laboratory tests are consistent with the theoretical results discussed above. For replicates which were split after mixing of the reference solution and sample, *R* ratios were typically 1.0 ± 0.06 (R.S.D., $n = 5$) and *U* ratios were typically 1.0 ± 0.10 (R.S.D., $n = 5$) for comparisons relative to 3-OH-B*a*P. Corresponding values for conventional ratios based only upon ^{14}C varied dramatically with R.S.D. values between 50–300%. All these results were somewhat worse than predicted by theory, perhaps due to interferences from other components and drifting chromatogram baselines. However, the marked advantages for the *R* and *U* ratio were corroborated by the empirical results.

These dual-label techniques are powerful for comparing reaction efficacies and should be adaptable to isotope-selective measurements such as emission spectrometry, mass fragmentometry and resonance spectroscopic methods, which would allow for use of non-radioactive compounds. Additionally, they could be easily used for non-biological reaction systems such as synthesis reactions and decompositions.

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