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Determination of the specific activity of sheep plasma amino acids using high-performance liquid chromatography: comparison study between liquid scintillation counter and on-line flow-through detector

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Abstract

A method was developed for the determination of the specific activities of leucine and phenylalanine in plasma using a flow-through scintillation counter coupled with high-performance liquid chromatography components. Results were compared with those obtained from liquid scintillation counting. Differences in the specific activities of leucine and phenylalanine between the two methods were not statistically significant. We concluded that flow-through radioactivity detection can be used for quantitative amino acid assays. However, the minimum activity that can be detected may be prohibitively low in certain applications.

Keywords: Leucine; Phenylalanine

1. Introduction

The use of labeled tracers has become common in the study of in vivo metabolism [1–5]. In these studies, tracer quantities of an isotopically labeled form of the substrate of interest (tracee) are infused into the organism and metabolic processes are inferred from the disappearance rate and dilution of the tracer. Metabolic fluxes are calculated from the rate of change of the tracer mass in an accessible compartment, usually blood plasma, and the rate of change of the tracer dilution by unlabeled tracee. Thus, accurate and precise determinations of both tracer and tracee concentrations are required.

Tracers labeled with ^{14}C or ^{3}H are widely used in metabolic studies [6–10]. Tracer concentration is expressed as radioactivity per ml, while tracee concentrations are expressed as mass units per ml; the specific activity (SA) is defined as the tracer to tracee ratio. It is crucial that the tracer and tracee concentrations be determined from the same plasma sample, assuring that any systematic bias introduced by sample preparation affects both measurements equally. This is difficult practically in studies of amino acid kinetics, as determination of concentration for most amino acids requires chromatographic separation in addition to either pre- or post-column derivatization. Thus, while the sample's radioactivity

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may be determined directly by scintillation counting, considerable sample manipulation is required for measurement of tracee concentration.

In order to assure that any errors in sample preparation or derivatization apply equally to radioactivity and concentration determinations, it is necessary therefore to measure the radioactivity in the derivatized sample. When using pre-column derivatization, the sample may be split and the radioactivity measured before chromatography, or the fraction of interest collected and the radioactivity measured subsequently. However, fraction collection is difficult to automate and often results in the sample of interest being contained in large eluent volumes. Thus, labor intensive evaporative sample concentration is required to achieve workable sample volumes for scintillation counting.

Recently, flow-through scintillation counters connected in series with other high-performance liquid chromatography (HPLC) components have been used for the determination of radioactivity [11–15]. They have been used most often in protein chemistry. These instruments have the advantages that a single sample is used for both concentration and radioactivity determinations and they are compatible with automated sample injection and data processing. The purpose of this study was to develop a method for determining the specific activity of free leucine and free phenylalanine in plasma using a flow-through scintillation counter, and to compare data obtained with this instrument with established methods of specific activity determination.

2. Experimental

The Picotag method of plasma amino acid analysis was performed as reported previously [16], with modifications as described below.

2.1. Reagents

Eluent 1, eluent 2, Picotag column (30 mm long, 3.9 mm I.D.) and Picotag diluent were purchased from Waters (Milford, MA, USA). Prior to use, the eluents were filtered and degassed for 2–3 min. Triethylamine (TEA) was obtained from Fisher Scientific (Itasca, IL, USA), phenylisothiocyanate (PITC) was from Pierce (Rockford, IL, USA), and the amino acids and 5-sulfosalicylic acid (SSA) were from Sigma (St. Louis, MO, USA). Acrodisc (0.45 μm pore size) filters were purchased from Gelman (Ann Arbor, MI, USA). L-[1-^{14}C]Leucine (0.01 *M* HCl solution) and L-[2,6-^{3}H]phenylalanine (aqueous solution containing 2% ethanol) were purchased from Amersham Life Science (Arlington Heights, IL, USA). Scintillation cocktail ''Flo-Scint IV'' was obtained from Packard Instruments (Meridian, CT, USA). High purity water from a NanoPure II system (Barnstead, Dubuque, IA, USA) was used for all purposes during the experiments.

2.2. Instrument set-up

The HPLC instrumentation (Waters Chromatographic division) consisted of a ''WISP'' auto-injector Model 710B, two Model 510 pumps, one Model 440 absorbance detector set at 254 nm, one system interface module (SIM) and a Model 820 maxima system controller. The column temperature was maintained at 46.0±0.1°C with a column heater of model COL. HTR. MOD.

The liquid scintillation counter (LSC) was from Beckman (Fullerton, CA, USA; LS 6000 IC/TA/LL). ScintiSafe Econo cocktail (Fisher Scientific) was used.

A Radiomatic Flo-One/beta Model A200 flow-through scintillation counter was connected in series with the UV detector, to determine the radioactivity in the column effluent. The size of the liquid flow cell was 2.50 ml. The HPLC flow-rate was 1.0 ml/min, while that of the scintillation pump from the scintillation detector was 4.0 ml/min. The update time was 6 s. The eluate from the HPLC column was mixed in a special chamber with a high efficiency non-gelling fluid (Flo-Scint IV) using the scintillation pump. The mixture was then pumped through the cell, which was positioned between two photomultiplier tubes, where the radioactivity was measured. The radioactivity data were collected and stored in the built-in hard drive of the detector. Since there were no significant eluent quenching effects throughout the run, as determined from the quench curve, the isocratic mode was used to calculate the dpm value. The efficiency of both channels (^{14}C and ^{3}H), along with the spill over percent of the ^{14}C

channel into the ^{3}H channel, were calculated prior to the experiment.

2.3. Amino acid sample preparation

Sheep plasma containing L-[1-^{14}C]leucine and L-[2,6-^{3}H]phenylalanine were obtained from ovine fetal metabolic studies [17,18]. Plasma samples (0.30–0.50 ml) were deproteinized with an equal volume of 4% SSA. The free amino acids in the plasma were separated from the α-keto acids and derivatized as previously reported [16]. The derivatized amino acids were dried in a rotary evaporator (Savant, NY, USA). The dried samples were then reconstituted with Picotag diluent and filtered through 4 mm Acrodisc filters (0.45 μm pore size). Volumes (60 μl) of these filtrates (derivatized amino acids) were used to determine the radioactivity of ^{14}C-Leu and ^{3}H-Phe using LSC. The same amount was also used to obtain the masses of amino acids from HPLC and the radioactivity values from the Flo-1 detector. The potential loss of radioactivity due to the derivatization process was accounted for by prepared standards of known radioactivity for each tracee of interest.

2.4. Compensation for concentration and dpm loss

A 2.0-ml amino acid standard solution containing 0.3750 m*M* Leu, and 0.1875 m*M* Phe (27 500 dpm/ml ^{14}C-Leu and 27 500 dpm/ml ^{3}H-Phe) was prepared. From this solution, 30, 60, 90, 120 and 150 μl volumes were counted in the Beckman LSC prior to derivatization. This will be referred to as 'pre-DHS' (pre-derivatized hot standard). The remaining amino acid standard solution (1550 μl) was processed with the deproteinized plasma amino acid samples using the same derivatizing reagent. The dried derivatized standards were reconstituted with the same amount (1550 μl) of Picotag diluent. This was then filtered and counted as 30, 60, 90, 120 and 150 μl volumes in the Beckman LSC. This will be referred to as ''DHS'' (derivatized hot standard).

To compare the dpm values of the samples obtained using the Flo-1 detector, the same volumes of the standards (30, 60, 90, 120 and 150 μl) were injected into the HPLC system. The masses of Leu and Phe were determined using the response factors of the UV peaks [19].

2.5. Statistical considerations

Data were evaluated using linear regression analysis and analysis of variance (ANOVA).

3. Results

Fig. 1a shows the radiochromatogram (obtained from the Flo-1 detector) of a standard solution containing known quantities of ^{14}C-Leu and ^{3}H-Phe. The first peak is for ^{14}C-Leu and the second one is for ^{3}H-Phe. Fig. 1b is that of a typical plasma sample from a catheterized fetal lamb. In each case, the peaks were well separated and showed no interference between ^{14}C and ^{3}H.

To account for the concentration and dpm loss of the samples during the derivatization process, the pre- and post-derivatization radioactivities of the standards were compared for ^{14}C-Leu and ^{3}H-Phe using both the LSC and the Flo-1 detector. The standard curves are summarized in Fig. 2a for ^{14}C-Leu and in Fig. 2b for ^{3}H-Phe. The results were very similar between the LSC and Flo-1 methods for each isotope, with correlation coefficients approaching unity. For both ^{14}C-Leu and ^{3}H-Phe, the slope of the line was steeper with a more negative intercept using the Flo-1 method compared to the LSC method, presumably reflecting differences in instrument sensitivities. The actual dpm of the plasma amino acids (^{14}C-Leu and ^{3}H-Phe) before derivatization were obtained using these standard curves and were used in further calculations.

Fig. 3 shows the mean SAs of leucine and phenylalanine for six different studies where ^{14}C-Leu and ^{3}H-Phe were infused in trace amounts into catheterized fetal lambs. The graph depicts the mean±S.E.M. of SAs obtained in metabolic studies at four time points after the start of isotopic infusion. The results are shown along with the standard error bars for each blood draw. The solid lines represent the specific activities for leucine and phenylalanine obtained using the LSC, whereas the dashed lines represent those using the on line Flo-1 detector. The experimental errors are within the typical error limits

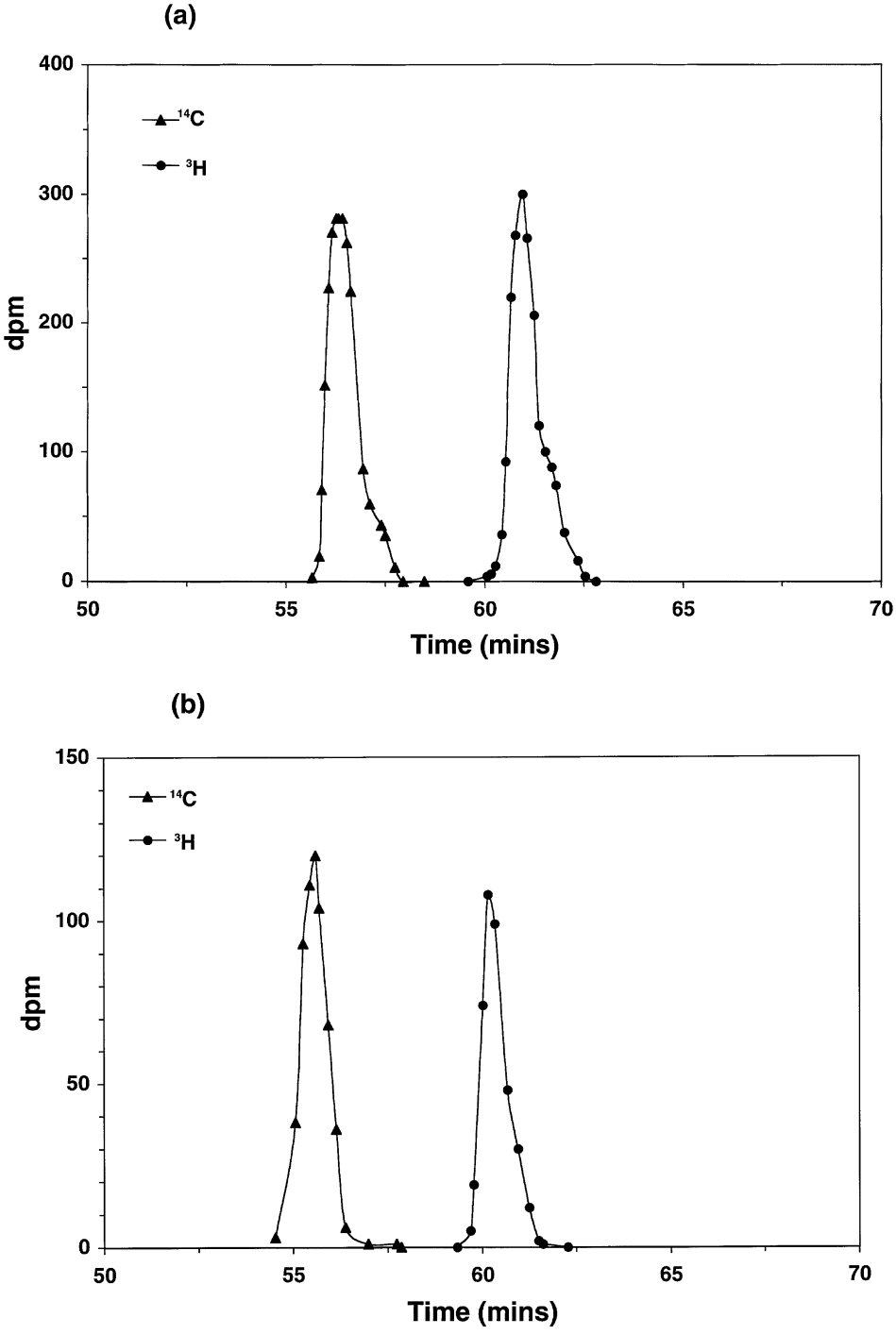

Fig. 1. (a) Radiochromatogram of a standard solution containing ^{14}C-leucine and ^{3}H-phenylalanine. (b) Radiochromatogram of a typical sample containing ^{14}C-leucine and ^{3}H-phenylalanine.

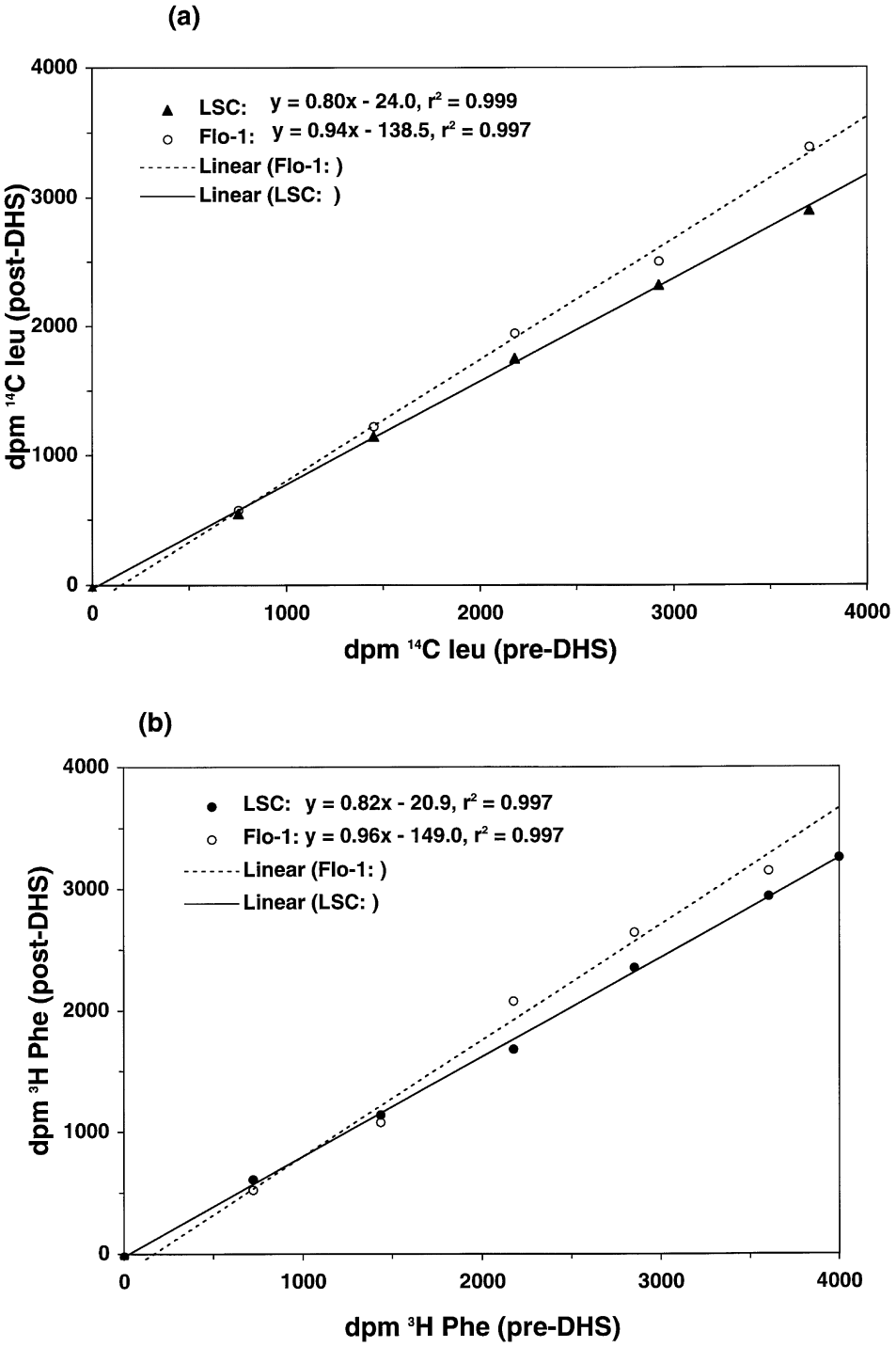

Fig. 2. (a) Activity (in dpm) of a ^{14}C-leucine standard before vs. after derivatization, as analyzed by the LSC and Flo-1 methods. (b) Activity (in dpm) of a ^{3}H-phenylalanine standard before vs. after derivatization, as analyzed by the LSC and Flo-1 methods.

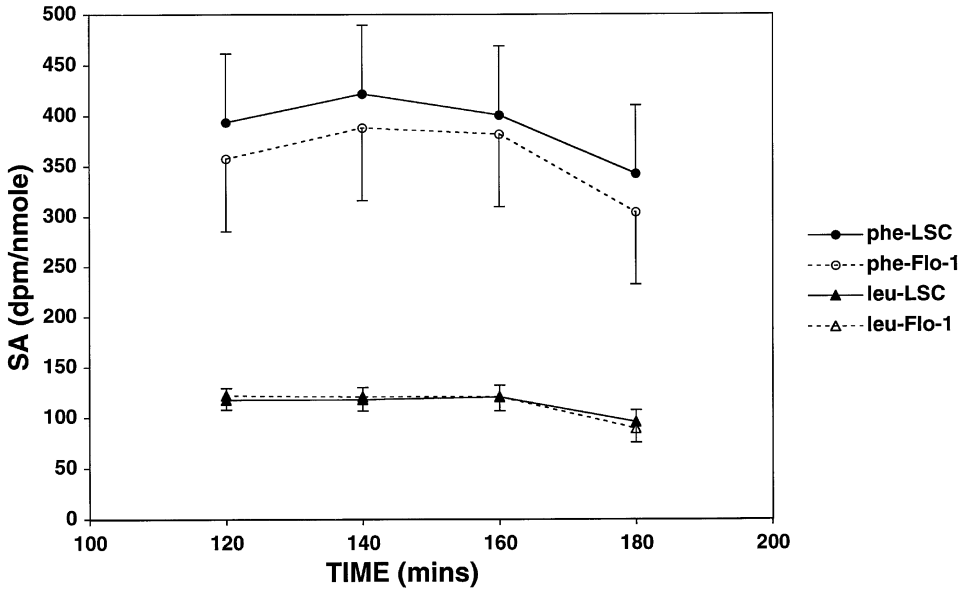

Fig. 3. Specific activity of leucine and phenylalanine as determined by LSC and Flo-1 from six studies with tracer-infused fetal lambs vs. the time (in min) after the isotope infusion. Each point is the mean of six studies. Error bars are standard error of the mean.

for these studies. Specific activities for leucine were 116±5.0 and 118±6.3 dpm/nmole, $p=0.97$ (all values are expressed as the mean±S.E.M.), obtained using the LSC and Flo-1 methods, respectively. For phenylalanine, these values were 397±26.1 and 366±25.4 dpm/nmole, $p=0.26$, for LSC and Flo-1, respectively. Differences in SA between the two methods for each isotope are not statistically significant.

Once the SA of the amino acid is known, the turnover rate or the rate of appearance of the amino acid can be calculated. The calculated turnover rates in these six studies are shown in Table 1. All are well within the range of turnover rates described by other investigators, either using radioactive or stable isotopic tracers [20–22].

We further defined a range of radioactivity that resulted in the least variance of the actual detected

Table 1
Comparison of turnover rates using LSC and Flo-1 methods

Study	Leu					Phe				
	SA (dpm/nmol)		$IR\cdot10^{-6}$ (dpm/min)	RA (mmol/min)		SA (dpm/nmol)		$IR\cdot10^{-6}$ (dpm/min)	RA (mmol/min)	
	LSC	Flo-1		LSC	Flo-1	LSC	Flo-1		LSC	Flo-1
1	105	105	3.63	34	34	398	412	4.26	11	10
2	105	113	3.46	33	31	469	416	4.58	10	11
3	171	192	3.29	19	17	665	623	4.46	7	7
4	126	116	3.55	28	31	237	221	2.83	12	13
5	107	101	3.17	30	32	352	293	3.59	10	12
6	84	82	3.18	38	39	264	230	3.51	13	15
Mean	116	118	3.38	30	30	397	366	3.87	14	11
S.E.	5.0	6.3	0.03	1.1	1.2	26.1	25.4	0.11	0.4	0.4

RA=IR/SA, where SA=specific activity, IR=infusion rate and RA=rate of appearance.

radioactivity from the theoretical 100% recovery level. Identical volumes of standard solutions of ^{14}C-Leu and ^{3}H-Phe were analyzed by the Flo-1 and LSC methods. The dpm obtained from the Flo-1 detector were then plotted against the dpm obtained from the LSC. These results are shown in Fig. 4a for ^{14}C-Leu and in Fig. 4b for ^{3}H-Phe. The solid lines in both figures represent the experimental results. The dashed line is the theoretical line for 100% detection. The region where the solid line and the broken line superimpose is considered to be the best working zone.

4. Discussion

The use of radioactive tracers in metabolic studies requires accurate laboratory analyses of the concentrations of both the isotopic tracer and tracee in the same sample. The technique used most often involves liquid scintillation counting in conjunction with the HPLC technique. Recently, flow-through scintillation counters have been connected in series with other HPLC components in order to measure both radioactivity and concentration in the same sample volume. In this study, we have compared the results obtained using both methods for the measurement of the specific activity of leucine and phenylalanine in plasma samples and we found that the two methods yield identical values for the SA of leucine and phenylalanine.

A significant issue using either method for determining the SA is the minimum radioactivity in the analyte that is needed to obtain reproducible results. Total sample radioactivity in dpm is a function of the SA, the tracee concentration and the sample size. The SA is a function of the infusion rate of tracer. Therefore, the total radioactivity can be increased by either increasing the sample size or the infusion rate of tracer. Both have drawbacks. In small animals, the available sample size is limited; increasing the tracer infusion rate significantly increases the cost of experiments, especially when using large animals. Therefore, determination of the minimum detection activity (MDA) is important.

MDA, or the minimal amount of radioactivity needed to obtain reproducible results, varies according to the width of the radiochromatogram peak, the residence time of the sample within the detection cell and the cell size. In fact, virtually all compounds behave differently under different chromatographic conditions.

Reich's equation [23] was used to compute the MDA for ^{14}C and ^{3}H:

$$\mathrm{MDA} = (B \times W)/(T \times E)$$

where B = background (in cpm), W = peak width (in min), T = residence time (in min; cell volume/total flow-rate), E = efficiency/100.

The MDA of a flow detector is not directly related to the total amount of activity in a given peak, but rather to the SA of any flow segment residing in the detector at any given time. Since B, W, and E are constants for a particular instrument and a particular assay, the only variable in the equation given above is T, the residence time. The residence time can be changed by varying the cell volume, the flow-rate, or both. An increase in cell volume or a decrease in flow-rate produces a decrease in the MDA. The total flow-rate is the sum of the flow-rates from the HPLC pump and that of the flow of scintillation cocktail fluid through the detector. There is a practical lower limit to the total flow-rate required to obtain a homogeneous sample. By increasing the cell volume to 2.50 ml and maintaining a flow-rate of 5.0 ml/min (1.0 ml/min of HPLC eluate and 4.0 ml/min of scintillation cocktail from the detector pump), we obtained MDAs for the present detector and for this particular assay of 350 and 1300 dpm for ^{14}C and ^{3}H, respectively. From these data, and knowledge of the approximate plasma amino acid concentration and the turnover rate, the appropriate tracer infusion rate can be calculated.

A new analytical method must be reproducible and yield results that are comparable to those obtained through other established methods. If these conditions are met, the new method will gain acceptance based on improved efficiency or decreased costs. In this study, we compared results using a liquid scintillation counter and an on-line HPLC flow-through detector to determine the specific activity of free amino acids in sheep plasma samples. The combination of HPLC and on-line scintillation counting saves time and presents considerable technical and economical advantages.

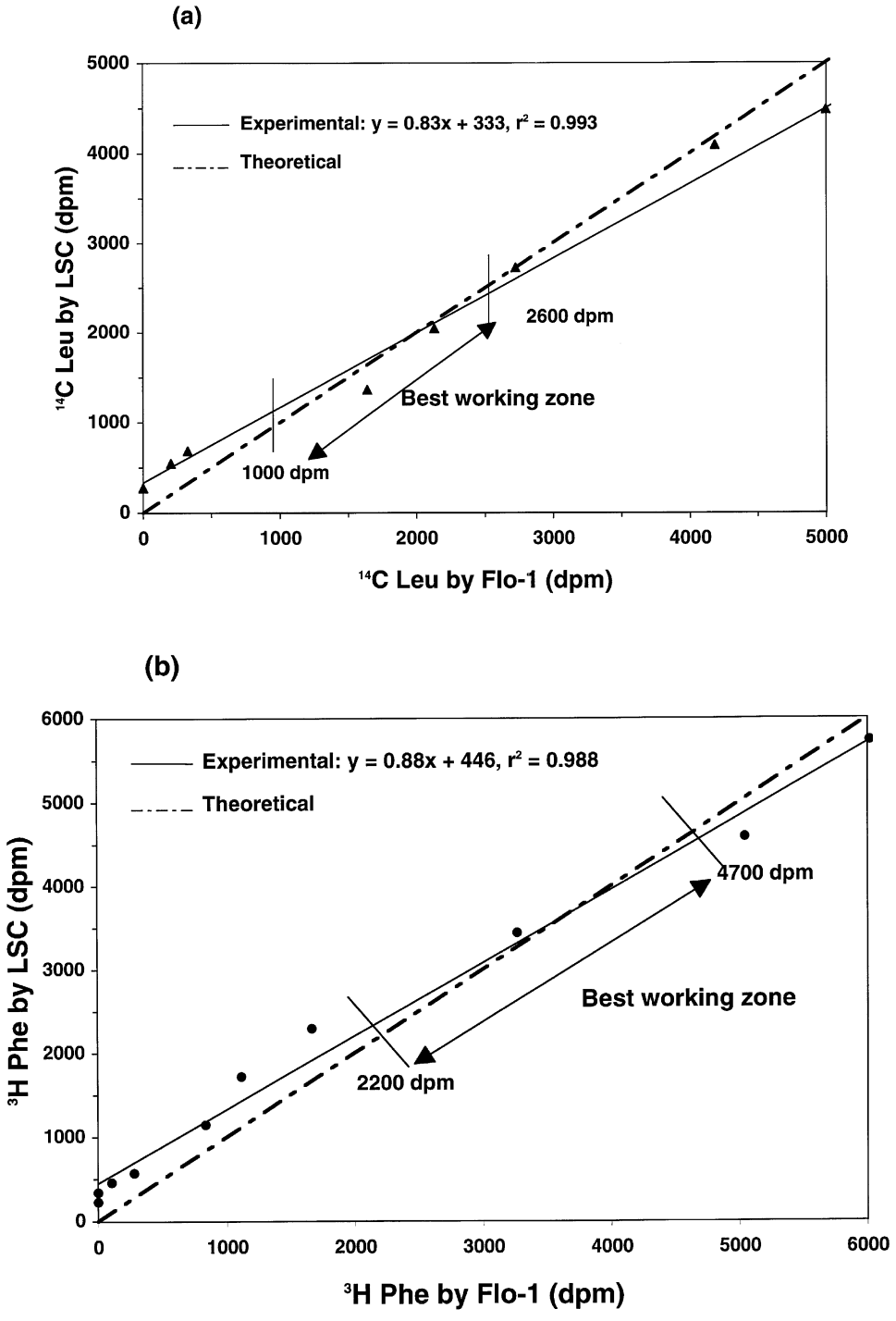

Fig. 4. (a) Comparison of the activity of ^{14}C-leucine as analyzed by the LSC and Flo-1 methods. (b) Comparison of the activity of ^{3}H-phenylalanine as analyzed by the LSC and Flo-1 methods.

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