2-24-2017

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Mohammad K. Miah
Texas Tech University Health Sciences Center

Ekram A. Chowdhury
Texas Tech University Health Sciences Center

Ulrich Bickel
Texas Tech University Health Sciences Center

Reza Mehvar
Chapman University, mehvar@chapman.edu

Recommended Citation

Miah MK, Chowdhury EA, Bickel U, Mehvar R. Evaluation of [14C] and [13C]sucrose as blood-brain barrier permeability markers. *J Pharm Sci.* 2017 Feb 23. pii: S0022-3549(17)30083-7. doi: 10.1016/j.xphs.2017.02.011.

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Mohammad K. Miah[1,a], Ekram A. Chowdhury[1,2], Ulrich Bickel[1,2,*], Reza Mehvar[1,2,3,*]

[1] *Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Texas, USA*

[2] *Center for Blood-Brain Barrier Research, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Texas, USA*

[3] *Department of Biomedical and Pharmaceutical Sciences, Chapman University, School of Pharmacy, Irvine, California, USA*

**Correspondence to:* Reza Mehvar, Ph.D., Department of Biomedical and Pharmaceutical Sciences, Rinker Health Science Campus, Chapman University, 9401 Jeronimo Road, Irvine, CA 92168, USA; Phone: (714) 516-5490; FAX: (714) 516-5481; E-mail address: mehvar@chapman.edu or Ulrich Bickel, Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Texas, USA; phone: 806.414.9236; E-mail: ulrich.bickel@ttuhsc.edu.

[a] Present Address: School of Pharmacy, University of Pittsburgh, Pittsburgh, PA, USA

ABSTRACT

Non-specific quantitation of [^{14}C]sucrose in blood and brain has been routinely used as a quantitative measure of the *in vivo* blood-brain barrier (BBB) integrity. However, the reported apparent brain uptake clearance (K_{in}) of the marker varies widely (~100 fold). We investigated the accuracy of the use of the marker in comparison with a stable isotope of sucrose ([^{13}C]sucrose) measured by a specific LC-MS/MS method. Rats received single doses of each marker, and the K_{in} values were determined. Surprisingly, the K_{in} value of [^{13}C]sucrose was 6-7 fold lower than that of [^{14}C]sucrose. Chromatographic fractionation after *in vivo* administration of [^{14}C]sucrose indicated that the majority of the brain content of radioactivity belonged to compounds other than the intact [^{14}C]sucrose. However, mechanistic studies failed to reveal any substantial metabolism of the marker. The water: octanol partition coefficient of [^{14}C]sucrose was > 2 fold higher than that of [^{13}C]sucrose, indicating presence of lipid-soluble impurities in the [^{14}C]sucrose solution. Our data indicate that [^{14}C]sucrose overestimates the true BBB permeability to sucrose. We suggest that specific quantitation of the stable isotope (^{13}C) of sucrose is a more accurate alternative to the current widespread use of the radioactive sucrose as a BBB marker.

Keywords: blood-brain barrier permeability, [^{14}C]sucrose, [^{13}C]sucrose, apparent brain uptake clearance

Introduction

One of the essential features of the blood-brain barrier (BBB) is the presence of complex intercellular tight junctions between adjacent endothelial cells,[1] which results in a very high transendothelial electrical resistance, estimated at ~1,800 Ω cm^2,[2] and severely restricts paracellular passage of polar compounds when compared to vascular beds in other organs.[3] Impairment of the BBB is recognized as either a pathophysiologic consequence or a cause of multiple disease states affecting the CNS, including traumatic brain injury, ischemic stroke, brain tumors, and neuroinflammatory, infectious, or neurodegenerative brain disorders.[4-8] Measurement of passive BBB permeability is therefore one of the most frequently used parameters in experimental and clinical studies.

Diverse markers ranging from vital dyes, such as Evans Blue,[9] and sugars, like sucrose and inulin,[10] to proteins, such as radiolabeled albumin[10,11] and horseradish peroxidase,[12] have been used in the BBB field in preclinical studies to determine the integrity of the barrier. For the quantification of subtle changes in paracellular permeability, no single ideal marker has emerged to date.[13] For example, as a hydrophilic small drug molecule, atenolol has been occasionally used to represent putative paracellular transport.[14-16] However, while atenolol is 99% charged at physiological pH, transcellular passage of the neutral form may account for a fraction of brain uptake measured *in vivo*.[17] In practical terms, a permeability marker should not have significant pharmacological effects, as it could alter physiological parameters. While not a drug itself, the disaccharide sucrose may be considered the most widely accepted standard for the precise measurement of paracellular BBB permeability.[18-22] This is because sucrose is uncharged, not subject to protein binding, metabolically stable after parenteral administration, falling into the molecular weight range of most small molecule drugs, and, to our knowledge, lacking

measurable membrane transport by active or facilitative mechanisms in vertebrates. With respect to *in vivo* studies, a recent review[13] on BBB markers rated radiolabeled sucrose as the only small molecular weight agent providing quantitatively accurate data. However, at least one earlier literature report[23] indicated that radiolabeled sucrose, especially when used in typical fashion with counting of just the whole radioactivity in blood and tissue samples (i.e. without chromatographic separation), may give spurious data due to presence of minor lipophilic contaminants.

To potentially replace radiolabeled tracers and circumvent the non-specificity of total radioactivity measurement associated with the use of [^{14}C]sucrose, we recently introduced a highly specific and sensitive non-radioactive technique for measurement of BBB permeability, based on LC-MS/MS detection of [^{13}C]sucrose.[24] In the current study, we conducted a rigorous side-by side comparison of the two isotopically labeled sucrose analogs after intravenous bolus injection in rats.

Materials and Methods

Chemicals and Reagents

We purchased [UL-$^{13}C_{12}$]sucrose (all the carbons in both glucose and fructose molecules are labeled with ^{13}C isotope; denoted [^{13}C]sucrose) and the internal standard, which was [UL-$^{13}C_6^{fru}$]sucrose (all the carbons in the fructose molecule are labeled with ^{13}C isotope), from Omicron Biochemicals (South Bend, IN, USA). [^{14}C]Sucrose, Solvable, and Hionic-Fluor solution were purchased from PerkinElmer (Boston, Massachusetts, USA). Specific activity of the [^{14}C]sucrose stock solution was 400-700 mCi/mmol. LC-MS grade water (J.T. Baker) was purchased from Avantor Performance Materials, Inc. (Center Valley, PA, USA). 1-Octanol was purchased from Alfa Aesar (Ward Hill, MA, USA). Analytical grade ammonium hydroxide and

LC-MS grade acetonitrile (ACN) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). For anesthesia, ketamine and xylazine solutions were purchased from Lloyd Laboratories (Shenandoah, IA, USA). Heparin solution was purchased from APP Pharmaceuticals (Schaumburg, IL, USA). All other chemicals were analytical grade and obtained from commercial sources.

Animals

Adult, male Sprague-Dawley rats were purchased from Charles River laboratory (Wilmington, MA) and acclimated in single, ventilated cages with 12-h dark-light cycles in a temperature- and humidity-controlled room with free access to the food and water. The average weight of animals was in the range of 379 – 415 g, and the CV of body weight in each group was < 5%.

All the animal procedures used in this study were approved by Texas Tech University Health Sciences Center's Institutional Animal Care and Use Committee and were consistent with the guidelines set by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

In Vivo BBB Permeability to [^{14}C] and [^{13}C]Sucrose

Rats were anesthetized with ketamine: xylazine, and catheters were placed in their penile vein and femoral artery for drug injection and serial blood sample collection, respectively. For [^{14}C]sucrose experiments, a single dose of 3 μCi (~ 5 μg/kg) was administered to different groups of animals, which were then euthanized 30, 60, or 240 min after the injection (n = 3/group). Serial blood samples were collected at different time points before euthanasia. At the end of sampling, a catheter was placed in the left ventricle and the whole body was perfused with ice-cold saline at a rate of 25 mL/min for 5 min, and the brain was collected. One hemisphere of

the brain was cut into small pieces, and 0.2-0.3 g of the brain tissue was added to 2 mL of Solvable to digest the tissue. Additionally, 20 µL of blood and plasma samples were added to 2 mL of Solvable. After 24 h at room temperature, 100 µL of hydrogen peroxide was added to each sample to remove any possible color quenching, and samples were subjected to liquid scintillation counting after adding 12 mL scintillation fluid.

For [^{13}C]sucrose, different groups of rats were euthanized at 15, 30, 60, 90, 120, or 240 min after injection of [^{13}C]sucrose at a dose of 10 mg/kg (n = 3/group). The purpose of the additional time points used for [^{13}C]sucrose (15, 90, and 120 min), compared with the [^{14}C]sucrose experiments, was to further characterize the kinetic profile of this newly developed marker, which showed a BBB permeability profile different from that of [^{14}C]sucrose. Serial blood samples and terminal brain were collected as described above for [^{14}C]sucrose. Before analysis, plasma samples were diluted 100 times with water, and brain was homogenized in ice-cold water at a ratio of 1:9 to obtain brain homogenate. The brain homogenate and diluted plasma samples were analyzed by the LC-MS/MS method as described below.

Fractionation of Plasma and Brain [^{14}C]Sucrose after In Vivo Administration

After an overnight fast, rats (n = 3) were anesthetized with a ketamine: xylazine mixture (80:8 mg/kg) via intramuscular injection, and [^{14}C]sucrose was injected at a dose of 100 µCi/animal (~ 170 µg/kg) via penile vein. During anesthesia, the body temperature of rats was maintained at 37^{O}C. One h after the sucrose injection, a blood sample was collected from a femoral artery catheter and plasma was separated. Additionally, a catheter was placed in the left ventricle of the heart, and whole body perfusion was conducted with ice-cold saline at a rate of 25 mL/min for 5 min. Subsequently, the brain was collected and snap-frozen in cold (-80^{O}C) iso-pentane. The whole brain was homogenized in ice-cold water (1:4) containing 0.03% sodium

azide. After centrifugation, 2 mL of the supernatant was condensed to ~200 µL under a nitrogen stream, followed by addition of 1800 µL of acetonitrile and water mixture (80:20), vortexed, centrifuged at 20000 *g* for 10 min, and the supernatant was collected. The supernatant was divided into two parts; one part was used for HPLC fractionation and radioactivity measurements of the individual fractions, whereas the other part was used to measure the total, unfractionated radioactivity in the sample.

For fractionation, the supernatant was injected onto an HPLC column (Unison UK-Amino; 150 mm x 2 mm, 3 µm; Imtakt, Portland, OR, USA), using an injection volume of 50 µL at a time. With a mobile phase of water: acetonitrile (20:80, v/v), run at a flow rate of 0.4 mL/min, sucrose retention time was 4.25 min. Column eluent was collected every 15 sec for 15 min. At the end of all 50 µL injections, the collected fractions at each time point were pooled and subjected to liquid scintillation counting along with the other portion of the supernatant. To rule out degradation of [^{14}C]sucrose during sample preparation, blank brain homogenates, obtained from drug-free rats, were subjected to the same sample preparation process after spiking with [^{14}C]sucrose concentrations as observed in the *in vivo* study.

Plasma samples (10 µL) were mixed with 90 µL of acetonitrile: water (80:20), vortexed, and centrifuged. Similar to the brain samples, the supernatant was divided into two parts (40 µL each) and subjected to HPLC fractionation or direct measurement of total radioactivity. Similar to the brain and plasma samples, radioactivity in aliquots of the dosing solution for each animal was measured directly, and in individual fractions after HPLC fractionation.

In Vitro Stability of [^{13}C]Sucrose in Liver and Brain Homogenates and Blood

Fresh, heparinized blood, collected from rats, and water (control) samples were spiked with [^{13}C]sucrose to achieve a concentration of 10 ng/mL (n = 3). Fresh drug-free liver and brain

samples were homogenized (1:9) in citrate-phosphate buffer (100 mM), pH 6.5. Tissue homogenates and citrate-phosphate buffer (Control) were spiked with [^{13}C]sucrose to achieve a final concentration of 10 ng/mL. The spiked samples were kept in a shaking water bath at 37^{O}C for 3 h, and serial samples (20 µL each) were collected and processed for LC-MS/MS analysis.

In Vitro Stability of [^{13}C]Sucrose in Brain Cell Culture

Brain co-cultures consisted of a mixture of mouse primary astrocytes, glial cells, and neurons after first passage, where cells were 50% confluent. Neurons were 10 days and astrocytes and glial cells were 17 days old. Total numbers of cells per well were 30,000-40,000 for astrocytes and neurons and 5,000-10,000 for microglial cells. Three different concentrations of [^{13}C]sucrose (2, 4, and 10 ng/mL) were added to different wells in a 12-well plate, containing either cells or the culture medium alone (n = 3). The plates were then incubated for 24 h in a CO_2 incubator, and serial samples (20 µL) were collected at different time points (0, 2, 12, and 24 h) and subjected to LC-MS/MS analysis.

In Vitro Stability of [^{14}C]Sucrose in Brain Homogenates

Fresh drug-free brain was homogenized (1:9) in citrate-phosphate buffer (10 mM), pH 6.5, and the resulting brain homogenates were spiked with 2500 dpm/mL (0.7 ng/mL) of [^{14}C]sucrose. This concentration was selected to be close to the brain concentrations observed after *in vivo* administration of 100 µCi of [^{14}C]sucrose in the above fractionation studies. The spiked homogenates were placed in a shaking water bath at 37^{O}C, and samples were taken at zero (baseline) and 3 h, and subjected to the same procedure described above for fractionation studies (i.e., addition of 0.03% sodium azide, centrifugation, 10-fold concentration of the supernatant, and precipitation of proteins with acetonitrile: water). Similarly, the final supernatant was then

subjected to both HPLC fractionation, followed by radioactivity measurements of the individual fractions, in addition to counting of the total, unfractionated radioactivity.

Partition Coefficients of [^{14}C] and [^{13}C]Sucrose

Partition coefficients [^{14}C] and [^{13}C]sucrose between 1-octanol and water were determined according to the established methods. Water is very slightly soluble in 1-octanol (1.64 mol/l). Therefore, to reduce any errors during partitioning experiments, equal volumes of 1-octanol and water were mixed together and kept overnight with continuous stirring at room temperature to pre-saturate 1-octanol with water. The water-saturated 1-octanol was used for the following studies.

[^{13}C] (100 μg/mL) or [^{14}C] (146000 dpm/mL or ~ 40 ng/mL) sucrose solutions (5 mL) were made in water and added to an equal volume of pre-saturated 1-octanol in a separating funnel. Subsequently, the contents of the funnel were mixed for 30 min in a rotary machine, and samples (500 μL) were taken from both the water and 1-octanol media for analysis. Additionally, the water part was separated and underwent a second and third partitioning by the addition of equal volumes of fresh 1-octanol, followed by shaking and sampling as described above. For [^{13}C]sucrose measurements, undiluted 1-octanol and 100 fold diluted water samples were analyzed by LC-MS/MS. For [^{14}C]sucrose measurements, samples were added to the scintillation fluid and subjected to scintillation counter.

In addition to determination of the partition coefficient of the [^{14}C]sucrose stock solution used in the animal studies, partition coefficient of a second stock of [^{14}C]sucrose was also determined to investigate the potential differences between different lots of the marker.

LC-MS/MS Analysis of [^{13}C]Sucrose

The LC-MS/MS method is described in a recent publication.[24] In brief, samples (20 µL), after proper dilution, were subjected to protein precipitation by the addition of 180 µL of acetonitrile: water (80:20), which contained IS. Chromatographic separation was then performed using an Acquity BEH amide column (50 mm x 3 mm, 1.7 µm) and an isocratic mobile phase of acetonitrile: water: ammonium hydroxide (72:28:0.1, v/v), run a flow rate 0.2 mL/min. Column temperature was maintained at 45^{o}C. The retention time of sucrose was ~2.5 min. The MRM was monitored in negative mode, and the transitions for [^{13}C]sucrose and IS were 353→92 m/z and 347→89 m/z, respectively.

As reported before,[24] the recovery of [^{13}C]sucrose from the brain homogenates at concentrations of 10 (94.0%) and 100 (100%) ng/mL was almost quantitative. However, additional recovery experiments at a brain homogenate concentration of 3 ng/mL (n = 5) were conducted in the current study to verify the recovery of the marker at concentrations observed in this study. Similar to the previous method,[24] the recovery was determined by comparison of peak areas of brain homogenates and aqueous solutions spiked with [^{13}C]sucrose and subjected to an identical sample preparation method. In agreement with the previous report,[24] the recovery (mean ± SD) at a brain homogenate concentration of 3 ng/mL was 102 ± 4%.

Liquid Scintillation Counting

All radioactive samples (brain, plasma, HPLC fractions) were measured in a Beckman LS6500 liquid scintillation counter with appropriate window settings for ^{14}C. Quench monitoring (H-number) and automatic quench correction was applied to convert counts per minute (cpm) into disintegrations per minute per gram tissue (dpm/g) or dpm/mL for brain and plasma samples, respectively.

Pharmacokinetic Analysis

To determine the BBB permeability to both markers in naïve animals, apparent brain uptake clearance (K_{in}) values were estimated for individual time groups using the following equation:[25]

$$K_{in} = \frac{C_{br}^{t_{last}}}{AUC_{plasma}^{0-t_{last}}} \quad (1)$$

where $C_{br}^{t_{last}}$ and $AUC_{plasma}^{0-t_{last}}$ are the amount of the marker in the terminal brain sample (in units of mass/g of brain) corrected for the marker presence in brain plasma and the area under the plasma concentration-time curve (AUC) from time zero to the last sampling time, respectively. The $AUC_{plasma}^{0-t_{last}}$ was estimated by the log-linear trapezoidal rule. Additionally, the K_{in} values were also estimated from the Patlak plot,[26] which is defined by the following equation:

$$C_{br}^{t_{last}}/C_{plasma}^{t_{last}} = K_{in} \times AUC_{plasma}^{0-t_{last}}/C_{plasma}^{t_{last}} + (V_p + V_0) \quad (2)$$

where $C_{plasma}^{t_{last}}$ is the terminal plasma concentration of the marker and V_p and V_0 are the volume of the plasma space (in the absence of brain vascular washout) and an additional hypothetical volume outside the brain parenchyma in which the marker may be rapidly distributed.

To allow comparison of data between the [^{13}C] and [^{14}C]sucrose, the mass of the markers in the blood and brain were corrected for the injected dose and expressed as percentage of injected dose (%ID).

Statistical Analysis

Statistical analysis of data was performed using Prism software (GraphPad Software, LaJolla, CA). Data with three or more groups were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons. Two-way ANOVA, followed by Bonferroni multiple

comparisons, was used for the comparison of AUC, $C_{br}^{t_{last}}$, and K_{in} values of [^{13}C] and [^{14}C]sucrose at 30, 60, and 240 min. For *in vitro* stability studies, the relationships between the concentration of the marker and time were analyzed using linear regression analysis. In all cases, a p value < 0.05 was considered significant. Data are presented as mean ± SD or individual values.

Results

Plasma Kinetics and Brain Disposition Profiles of [^{13}C] and [^{14}C]Sucrose

The plasma concentration-time profiles of [^{13}C] and [^{14}C]sucrose in different groups of animals, which were euthanized at different time points (15-240 min), are presented in Fig. 1. Both markers showed similar profiles with an apparent multiexponential decline in their plasma concentrations. Additionally, the respective area under the plasma concentration-time curve from time zero to the last sampling time ($AUC_{plasma}^{0-t_{last}}$), terminal brain concentrations $C_{br}^{t_{last}}$, and apparent brain uptake clearance (K_{in}) values are presented in Table 1. As expected, the $AUC_{plasma}^{0-t_{last}}$ values generally increased with an increase in the sampling time for both markers (p < 0.01, Table 1). The plasma concentration-time course (Fig. 1b) and AUC values (Table 1) of [^{13}C] and [^{14}C]sucrose for the 30 min groups were very similar and not significantly different from each other. Although there was a trend towards higher plasma concentrations (Fig. 1c and Fig. 1f) and AUC values (Table 1) of [^{14}C]sucrose for the 60 min and 240 min groups, the differences between the AUC values of the two markers were not statistically significant (Table 1).

In contrast to a significant time-dependent increase in the AUC of both markers (Table 1), the terminal brain concentrations of both [^{13}C] and [^{14}C]sucrose remained relatively

unchanged ($P > 0.05$) during the 15-240 min sampling period for [^{13}C]sucrose and the 30-240 min sampling period for [^{14}C]sucrose (Table 1). Additionally, despite similar $AUC_{plasma}^{0-t_{last}}$ values for [^{13}C] and [^{14}C]sucrose, the brain concentrations of [^{14}C]sucrose were, on average, 7-10 fold higher than those of [^{13}C]sucrose at the same time intervals (Table 1). In contrast to the relatively constant brain concentrations, there was a progressive decline in the K_{in} values with an increase in the sampling time for both [^{13}C] ($p < 0.0001$) and [^{14}C] ($p < 0.01$) sucrose (Table 1). Of particular note is the finding that the K_{in} values for [^{14}C]sucrose were, on average, 6.3 (30 min group), 6.4 (60 min group), or 7.6 (240 min group) fold higher than those for [^{13}C]sucrose at the corresponding sampling time ($p < 0.0001$, Table 1). Whereas the K_{in} values ranged from 0.182 to 0.382 μL/(min.g) for [^{14}C]sucrose, the values for [^{13}C]sucrose ranged from 0.0241 to 0.0801 μL/(min.g) (Table 1).

The Patlak plots for [^{13}C] (Figs. 2a and 2b) and [^{14}C] (Figs. 2c and 2d) sucrose are depicted in Fig. 2. These plots were constructed using individual animal data for all the time points (Figs. 2a and 2c) or after exclusion of the 240-min groups (Figs. 2b and 2d). As demonstrated, exclusion or inclusion of the 240-min group did not substantially affect the intercepts or slopes of the plots for either [^{13}C] or [^{14}C]sucrose (Fig. 2). The K_{in} values estimated from the slopes of the Patlak plots were 0.0229 and 0.173 μL/(min.g) for [^{13}C] (Fig. 2a) and [^{14}C] (Fig. 2c) sucrose, respectively, indicating a 7.6 fold higher K_{in} for [^{14}C]sucrose when compared with the corresponding value for [^{13}C]sucrose. Similarly, the intercept of the line, which is an indication of a residual volume, was 4.6 fold higher for [^{14}C]sucrose (11.7 μL/g) than that for [^{13}C]sucrose (2.54 μL/g) (Fig. 2).

Fractionation of Brain and Plasma Samples after In vivo Administration of [^{14}C]Sucrose

Figure 3 depicts the chromatograms of three individual brain samples (Figs. 3a-3c), which were collected 1 h after the *in vivo* administration of [^{14}C]sucrose, along with the chromatograms of their respective dosing solutions. Additionally depicted in the figure are the chromatograms of a blank brain homogenate spiked *in vitro* with [^{14}C]sucrose (Fig. 3d) and a representative terminal plasma sample collected 1 h after the *in vivo* administration of [^{14}C]sucrose (Fig. 3e). As demonstrated in Fig. 3, whereas the dosing solutions (Figs. 3a-c), the *in vitro* spiked brain homogenate (Fig. 3d), and the *in vivo* plasma sample (Fig. 3e) showed a single peak related to sucrose at ~4.5 min, the chromatograms of the *in vivo* brain samples contained several prominent peaks, in addition to the peak expected at the sucrose retention time (Figs. 3a-3c). In fact, most of the total peak areas in the chromatograms of *in vivo* brain samples could be attributed to the retention times other than that for the sucrose peak (Figs. 3a-3c). We also measured the total radioactivities of the brain samples in the unfractionated samples to determine the recovery of the radioactivity from the fractionation procedure. The extent of recovery for the three samples was 95.0 ± 7.5%, indicating the chromatograms represent an almost complete recovery of the radioactivity in the brain samples.

Stability of [^{13}C]Sucrose in Biological Samples

To determine the possibility of *in vivo* metabolism of [^{13}C]sucrose, the stability of the marker in various biological media was tested *in vitro* at 37^{o}C. To avoid saturation of metabolism, the concentrations of [^{13}C]sucrose were selected to be much lower than the *in vivo* concentrations achieved after intravenous injection of [^{13}C]sucrose (10 ng/mL ≈ 0.00025 %ID per mL; 4 ng/mL ≈ 0.0001 %ID/mL, and 2 ng/mL ≈ 0.00005 %ID/mL). Figure 4 demonstrates the concentration-time courses of [^{13}C]sucrose in brain homogenate (Fig. 4a), brain cell co-

culture (Fig. 4b), liver homogenate (Fig. 4c), and blood (Fig. 4d). There were no significant changes in the concentrations of the marker in any of the biological samples tested as the slopes of the regression lines for all the biological samples were not significantly ($p > 0.05$) different from zero (Fig. 4).

Also shown in Fig. 4 are the chromatograms of brain homogenates before (time zero) and 3 h after incubation of a low concentration (2500 dpm or 0.7 ng per mL) of [^{14}C]sucrose at 37^{o}C, followed by HPLC fractionation of the samples (Fig. 4e). As demonstrated, both chromatograms showed a single major peak at the expected retention time of sucrose (4.25 min), which were virtually superimposable for the zero and 3 h samples, indicating no noticeable metabolism of [^{14}C]sucrose during the incubation period. Finally, the recovery of [^{14}C]sucrose from the HPLC fractions, relative to the unfractionated samples, was quantitative, indicating complete recovery of the radioactivity form the HPLC column during the 15 min run.

Octanol: Water Partition Coefficient of [^{13}C] and [^{14}C]Sucrose

The octanol: water partition coefficient (K_p) values of [^{13}C] and [^{14}C]sucrose after three successive partitioning procedures are presented in Fig. 5. The K_p values of [^{13}C]sucrose after the first ($2.75 \times 10^{-4} \pm 0.07 \times 10^{-4}$), second ($2.66 \times 10^{-4} \pm 0.21 \times 10^{-4}$), and third ($2.63 \times 10^{-4} \pm 0.29 \times 10^{-4}$) experiments were almost identical ($p > 0.05$) (Fig. 5a). However, the K_p values of [^{14}C]sucrose after the second ($4.67 \times 10^{-4} \pm 0.41 \times 10^{-4}$) and third ($4.32 \times 10^{-4} \pm 0.26 \times 10^{-4}$) experiments were significantly ($p < 0.01$ and < 0.001, respectively) lower than that after the first experiment ($5.74 \times 10^{-4} \pm 0.40 \times 10^{-4}$) (Fig. 5b). Furthermore, the K_p value of [^{14}C]sucrose (Fig. 5b) after the first experiment was more than two-fold higher than the corresponding value for [^{13}C]sucrose (Fig. 5a). Even after the third partitioning experiment, the K_p value of [^{14}C]sucrose (Fig. 5b) remained 60% higher than the corresponding value for [^{13}C]sucrose (Fig. 5a). The

partition coefficient values for a second lot of [^{14}C]sucrose (Fig. 5c), which was not used in the studies reported here, were very similar to those of the lot used in the current studies (Fig. 5b) in terms of both absolute values and pattern after successive partitioning.

Discussion

The search for ideal markers for the *in vivo* assessment of BBB integrity has been elusive. However, radiolabeled small molecules, such as sucrose and inulin, are believed to be one of the most appropriate groups of markers for the evaluation of the BBB integrity in pathological conditions.[13] In particular, radiolabeled [^{14}C]sucrose has been extensively used for quantitative determination of BBB integrity in many *in vitro*,[27-30] in situ brain perfusion,[31-36] and *in vivo*[11,18-22,37-45] studies. This is because sucrose is a water-soluble molecule with no significant metabolism after parenteral injection, no binding to plasma or tissue proteins, and very low permeability across the intact BBB. However, the literature values for the *in vivo* permeability of intact rat BBB to [^{14}C]sucrose, measured as the K_{in} value, vary widely, making it difficult to determine the true permeability of the BBB to the marker.

Two different methods have been used in the literature for the determination of the *in vivo* apparent brain influx clearance (K_{in}) of sucrose.[25] The single-brain sample method uses one group of animals to determine the terminal brain concentration and the plasma AUC of the marker over a defined sampling time (Equation 1). The multiple-brain sample method is based on the Patlak plot (Equation 2), which requires similar data obtained from multiple groups of animals with terminal brain samples taken at multiple times after the marker dosing. In the current study, we employed both methods for the estimation of K_{in} using both [^{14}C] and [^{13}C]sucrose markers (Table 1 and Fig. 2). Our results show that the single-brain sample K_{in}

values decline with time for both markers (Table 1), and the values for the longest tested sampling time (240 min) are close to the corresponding values obtained from the Patlak method (Fig. 2). The K_{in} values obtained from the slope of the Patlak plot are theoretically more accurate as any intercept arising from the residual blood contamination in the brain and/or distribution of sucrose to a fast-equilibrating space in the brain (represented by V_0 in Equation 2)[26] does not contribute to the K_{in} value.

The estimation of K_{in} using Equation 1 and Equation 2 are based on the assumption that brain is a compartment separate from the plasma and other tissues, receiving the marker through a unidirectional transfer from the plasma.[25,26,46] For very low permeability markers, such as sucrose, assuming unidirectional or bidirectional transfer would essentially yield the same results if sampling time is relatively short (within minutes to few hours). This is because the main determinant of the brain concentration is the rate of entry of the marker into the brain from the plasma, where the concentrations are much higher than those in the brain (for [^{13}C]sucrose: 500 and 100 fold at 15 min and 2 h, respectively; Fig. 1 and Table 1). The rate of back transfer of the marker from the brain to plasma would be negligible during the time when plasma concentrations are much higher than those in the brain. Using a two-compartment model with plasma and brain as separate compartments, Ohno et al.[20] predicted that the brain concentrations of sucrose would reach its maximum around 60 min after an intravenous dose of the tracer, with a very slow rate of decline afterward.[20] However, this model did not include the additional, fast-equilibrating space in the brain (represented by V_0 in Equation 2), which was suggested by Patlak et al.[26] In our studies, we observed relatively constant brain concentrations for both [^{13}C] and [^{14}C]sucrose during the entire sampling period (15 min to 4 h) (Table 1). Therefore, our data is in agreement with the presence of the fast-equilibrating space in the brain[26] and/or contamination of

our brain samples with residual blood, which both prominently add to the total brain concentrations of the markers at the earlier time points, thus overestimating the brain concentrations and K_{in} values at those single-brain sample points (Table 1).

Regardless of the experimental design, however, the K_{in} values obtained for the radioactive [^{14}C]sucrose were 6.3-7.6 fold higher than those for [^{13}C]sucrose (Table 1 and Fig. 2). This surprising finding prompted us to conduct brain fractionation studies, which revealed that, indeed, most of the radioactivity in the brain after the *in vivo* administration of [^{14}C]sucrose was attributable to compounds other than the intact sucrose molecule (Fig. 3). In fact, if one could accurately estimate and use the radioactivity related to the sucrose peak only (Figs. 3a-c) in calculation of K_{in} values for [^{14}C]sucrose , the 6-7-fold difference in the K_{in} values between ^{14}C- and [^{13}C]sucrose would likely disappear.

Although it is generally believed that sucrose does not enter cells and is not subject to significant metabolism after intravenous administration, the possibility of minor metabolism of the marker in the liver by a very low activity sucrase enzyme has been raised.[47] Additionally, other investigators have shown that α-glucosidase activity, which breaks down starch and disaccharides to glucose, is also present in brain tissue.[48] Therefore, based on the fractionation studies (Fig. 3), we initially hypothesized that the intact [^{14}C]sucrose entered into the brain is subsequently metabolized in the brain. However, our *in vitro* studies with [^{13}C]sucrose in the brain homogenates (Fig. 4a) and brain cell co-cultures (Fig. 4b) did not support this hypothesis.

Although we tested the *in vitro* metabolism of [^{13}C]sucrose in brain at concentrations that were much lower (Figs. 4a and 4b) than those obtained after *in vivo* administration of the marker (Table 1), these concentrations were still much higher than the *in vivo* brain concentrations of [^{14}C]sucrose (Table 1). Therefore, it might be argued that metabolism of

sucrose at [^{13}C]sucrose concentrations used in our *in vitro* studies (Figs. 4a and 4b) is saturated. Consequently, an additional experiment was conducted to test the metabolism of [^{14}C]sucrose at very low brain concentrations, similar to those observed after our *in vivo* experiments (Figs. 3a–3c). The results of these experiments at low [^{14}C]sucrose concentrations (Fig. 4e) were in agreement with those at higher concentrations of [^{13}C]sucrose (Fig. 4a), which showed absence of any noticeable *in vitro* metabolism of sucrose in the brain homogenate. Furthermore, *in vitro* spiking of the brain homogenate and subjecting it to the sample preparation method used in the fractionation study did not reveal any additional peaks other than the intact [^{14}C]sucrose (Fig. 3d). Therefore, presence of substantial peaks appearing in the brain chromatograms after *in vivo* administration of [^{14}C]sucrose (Figs. 3a-c) cannot be attributed to a potentially significant metabolism of [^{14}C]sucrose in the brain.

Alternatively, [^{14}C]sucrose could be metabolized in the periphery and the metabolites enter the brain. However, *in vitro* studies in the liver (Fig. 4c) and blood (Fig. 4d) did not reveal any substantial peripheral metabolism of the marker. Nevertheless, our studies do not rule out a very minor peripheral metabolism of sucrose to highly BBB permeable metabolites. Although the apparent K_{in} value of [^{14}C]sucrose, traditionally obtained by counting total radioactivity of brain and plasma samples without fractionation, is susceptible to error by a potential peripheral metabolism, the specific measurement of [^{13}C]sucrose by LC-MS/MS method is devoid of such potential problems.

A previous study[23] showed that old stocks of [^{14}C]sucrose produce higher K_{in} values when compared with new stocks of the marker. Based on the Patlak plots, the K_{in} values for new, 4-year-old, and 7-year-old stocks of [^{14}C]sucrose showed K_{in} values across different regions of the brain in the ranges of 0.072-0.14, 0.31-0.38, and 0.50-0.58 μL/(min.g), respectively. It was

speculated that these differences were due to presence of impurities in the older stocks of [^{14}C]sucrose, which could readily cross the BBB. Our stock solution of [^{14}C]sucrose was new and the Patlak plot of [^{14}C]sucrose data in our studies (Fig. 2c) resulted in a K_{in} value of 0.173 μL/(min.g), which was close to the values reported by Preston et al.[23] for their new stock. Therefore, we first concentrated on other possibilities, such as the *in vivo* metabolism of [^{14}C]sucrose. However, after failing to demonstrate any appreciable metabolism of [^{14}C]sucrose (Fig. 4), we hypothesized that even a new stock solution of [^{14}C]sucrose might have small quantities of BBB permeable impurities, which could result in the observed peaks in the brain chromatograms (Figs. 3a-c) and an overestimation of the true K_{in} value. Indeed, our successive water: octanol partitioning experiments, demonstrating higher K_p value for [^{14}C]sucrose, compared with that of [^{13}C]sucrose, clearly show that the lipid solubility of the two marker solutions are not similar as expected. These experiments are in agreement with the presence of lipid soluble impurities in the [^{14}C]sucrose solution, which are partially removed by successive partitioning (Fig. 5). Collectively, these data suggest that the 6-7 fold higher K_{in} values observed in our studies for [^{14}C]sucrose, compared with [^{13}C]sucrose, is due to the presence of BBB permeable impurities in the [^{14}C]sucrose stock solution combined with the non-specific radioactive counting of the brain samples. Therefore, the [^{13}C]sucrose K_{in} values obtained by the specific LC-MS/MS method of quantitation should represent a more accurate estimation of the BBB permeability to sucrose.

In addition to the extent of the radioactive impurities, the nature of impurities and their BBB permeability properties are important factors that could affect the outcome of studies with [^{14}C]sucrose. For instance, Preston et al.[23] showed that whereas the new and 4-year-old stock of [^{14}C]sucrose showed the same percentage of impurities, the older stock resulted in 3.5 fold

higher K_{in} values. Therefore, presence of even a small percentage of a highly BBB permeable impurity may drastically increase the estimated K_{in} value of [^{14}C]sucrose using the traditional nonspecific, total radioactivity counting method. Based on the above considerations, it appears also plausible that the true K_{in} values of other BBB permeability markers may be substantially lower than those reported in the literature, at least when used in form of tracers labeled with radioisotopes. The polysaccharide inulin may serve as a case in point. It is considered a hydrophilic marker of passive permeability with an average molecular weight of 5,000 Da, about 15 times the molecular weight of sucrose. The estimated K_{in} of [^{3}H]inulin ranged between 0.044 and 0.062 μL/(min.g), which was close to half the K_{in} of [^{14}C]sucrose measured in parallel in the same study.[19] However, that inulin K_{in} value is 2-3 fold higher compared to the K_{in} of 0.0229 μL/(min.g) as determined in our present experiments for [^{13}C]sucrose. These data suggest that, like [^{14}C]sucrose, the previously reported K_{in} values for [^{3}H]inulin are also an overestimation of the true BBB to inulin.

Radioactive sucrose in any format (^{14}C or ^{3}H-labeled) is unsuitable for clinical use. In contrast, nonradioactive sucrose is nontoxic as a nutrient, and it is an ingredient in parenteral drug formulations (e.g., iron sucrose and some preparations of IVIG). From that perspective, [^{13}C]sucrose could potentially be used in humans as a marker of BBB permeability. An example is brain microdialysis sampling, which is applied for metabolic monitoring in neurointensive care of patients suffering traumatic brain injury or subarachnoid hemorrhage[49] and in clinical studies in glioblastoma patients.[50] Given the sensitivity and accuracy of the analytical measurement of [^{13}C]sucrose by LC-MS/MS, quantification in microdialysate samples after systemic administration (e.g., intravenous bolus or infusions) could be used to monitor BBB permeability in humans.

There are variations in experimental designs in the literature for the *in vivo* determination of K_{in} values. For example, whereas some investigators remove the residual blood form the brain by in situ perfusion at the terminal sampling time, others use brain vascular markers, such as radiolabeled albumin, to estimate the contribution of brain blood to the brain concentrations of the marker. Additionally, as mentioned above, some investigators use the single-brain method while others use the multiple-brain (Patlak) method. Therefore, the question becomes what experimental design would likely yield the most accurate estimate of the true K_{in} value of sucrose. Based on our findings here, we propose to use the [^{13}C]sucrose marker in at least two groups of animals with different sampling intervals (such as 1 and 2 h), followed by removal of the residual blood, as much as possible using the in situ perfusion of the brain, and construction of the Patlak plot. Alternatively, if only one group of animals is used, we suggest a longer sampling time (such as 2 h) to reduce the error associated with single-brain sample method at earlier time points.

Conclusions

In conclusion, our data indicate that the use of [^{14}C]sucrose as a marker of BBB permeability might result in a substantial overestimation of the true BBB permeability to sucrose. This is mainly due to the non-specific method of quantitation of the total radioactivity in the brain, which might contain substantial amounts of radioactive compounds other than the intact marker. We suggest that the LC-MS/MS quantitation of the stable isotope (^{13}C) of sucrose is a more accurate alternative to the current widespread use of the radioactive sucrose as a BBB marker.

Acknowledgments

The Authors would like to thank Drs. Vardan Karamyan and Raktima Bhattacharya from Texas Tech University School of Pharmacy for preparation and conduct of brain co-culture study and assistance with the radiolabelled studies, respectively. Financial support for this study was provided by the Center for Blood–Brain Barrier Research at Texas Tech School of Pharmacy.

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Table 1

Plasma AUC, Brain Terminal Concentrations ($C_{br}^{t_{last}}$), and Brain K_{in} Values (Mean ± SD) of [^{13}C] and [^{14}C]Sucrose in Rats

Sampling Interval min	$AUC_{plasma}^{0-t_{last}}$ %ID.min/mL		$C_{br}^{t_{last}}$ %ID/g		K_{in} µL/(min.g)	
	[^{13}C]Sucrose	[^{14}C]Sucrose	[^{13}C]Sucrose	[^{14}C]Sucrose	[^{13}C]Sucrose	[^{14}C]Sucrose
15	13.2 ± 5.0	–	0.00102 ± 0.00020	–	0.0801 ± 0.0127	–
30	20.2 ± 0.7	21.4 ± 5.4	0.00122 ± 0.00008	0.00830 ± 0.00289***	0.0605± 0.0057	0.382 ± 0.055****
60	22.3 ± 4.8	32.0 ± 5.9	0.000872 ± 0.000136	0.00836 ± 0.00121***	0.0410 ± 0.0132^{a}	0.263 ± 0.015^{****,b}
90	33.2 ± 8.3^{a}	–	0.000994 ± 0.000248	–	0.0306 ± 0.0071^{a,b}	–
120	29.8 ± 3.7^{a}	–	0.000882 ± 0.000257	–	0.0295 ± 0.0068^{a,b}	–
240	35.7 ± 7.4^{a}	44.7± 12.2^{b}	0.000849 ± 0.000157	0.00814 ± 0.00221***	0.0241 ± 0.0026^{a,b}	0.182 ± 0.044^{****,b}

^{a}Significantly different from the respective 15-min group based on one-way ANOVA, followed by Tukey's post-hoc analysis.

^{b}Significantly different from the respective 30-min group based on one-way ANOVA, followed by Tukey's post-hoc analysis.

$^{***}p < 0.001$, $^{****}p < 0.0001$: Significantly different from the [^{13}C]sucrose value at the same sampling interval based on two-way ANOVA, followed by Bonferroni post-hoc analysis.

FIGURE LEGENDS

Figure 1. Plasma concentration-time courses of [^{13}C] (a-f) and [^{14}C] (b, c, and f) sucrose in rats. Different groups of animals (n = 3/group) received a single intravenous dose of [^{13}C]sucrose (10 mg/kg) or [^{14}C]sucrose (3 μCi). Serial blood samples were taken over 15 (a), 30 (b), 60 (c), 90 (d), 120 (e), or 240 (e) min for [^{13}C]sucrose or over 30 (b), 60 (c), or 240 (f) min for ^{14}C-sucrose. Symbols and bars represent mean and SD values, respectively.

Figure 2. Patlak plots of [^{13}C] (a and b) and [^{14}C] (c and d) sucrose in rats, including (a and c) and excluding (b and d) the 240 min group data. Different groups of animals (n = 3/group) received a single intravenous dose of [^{13}C]sucrose (10 mg/kg) or [^{14}C]sucrose (3 μCi). Serial blood samples were taken over 15, 30, 60, 90, 120, or 240 min for [^{13}C]sucrose or over 30, 60, or 240 min for [^{14}C]sucrose. Terminal brain samples were also collected after blood removal. Symbols represent individual animals.

Figure 3. HPLC fractionation of brain samples collected 1 h after the *in vivo* administration of [^{14}C]sucrose (a, b, and c), a blank brain homogenate spiked *in vitro* with [^{14}C]sucrose (d), and a representative plasma sample collected after the *in vivo* administration of [^{14}C]sucrose (e). For comparison, the fractionation of dosing solutions is also shown along with each brain sample (a-c). For *in vivo* experiments, a single intravenous dose (100 μCi) of [^{14}C]sucrose was injected into the rats (n = 3), and brain (after blood removal) and plasma samples were collected at 1 h after dosing.

Figure 4. *In vitro* stability of [^{13}C]sucrose in brain homogenate (a), brain cell co-culture (b), liver homogenate (c), and blood (d) and that of [^{14}C]sucrose in brain homogenate (e). [^{13}C]Sucrose was incubated at 37^{o}C for 180 min (brain homogenate, liver homogenate, and blood) or 24 h

(brain cell co-culture), and serial samples were collected. For comparisons, the time courses of [^{13}C]sucrose concentrations in the buffer or medium are also included. The brain (a) and liver (c) homogenate experiments were conducted simultaneously using the same buffer control. The concentrations of [^{13}C]sucrose were selected to be much lower than the *in vivo* concentrations achieved after intravenous injection of [^{13}C]sucrose (10 ng/mL ≈ 0.00025 %ID per mL; 4 ng/mL ≈ 0.0001 %ID/mL, and 2 ng/mL ≈ 0.00005 %ID/mL). Symbols and bars represent mean and SD values (n = 3), respectively, and the lines represent linear regression analysis of data. For [^{14}C]sucrose (e), brain homogenate samples were spiked with 2500 dpm or 0.7 ng per mL of the marker and incubated at 37^{o}C for 3 h. Subsequently, samples were taken at zero (before incubation) and 3 h and subjected to HPLC fractionation before counting. Also included in Fig. 4e are chromatograms of blank (vehicle) and tracer solutions.

Figure 5. Octanol: water partition coefficient (K_p) of [^{13}C] (a) and [^{14}C] (b) sucrose (n = 5/marker), which were used for in the current *in vitro* and *in vivo* studies, and a second lot of [^{14}C]sucrose (c), which is demonstrated for comparative purposes (n = 3). Aqueous samples were successively partitioned for 3 times. Symbols and horizontal lines represent individual and mean values, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$ based on one-way ANOVA, followed by Tukey's post-hoc analysis.

Figure 1

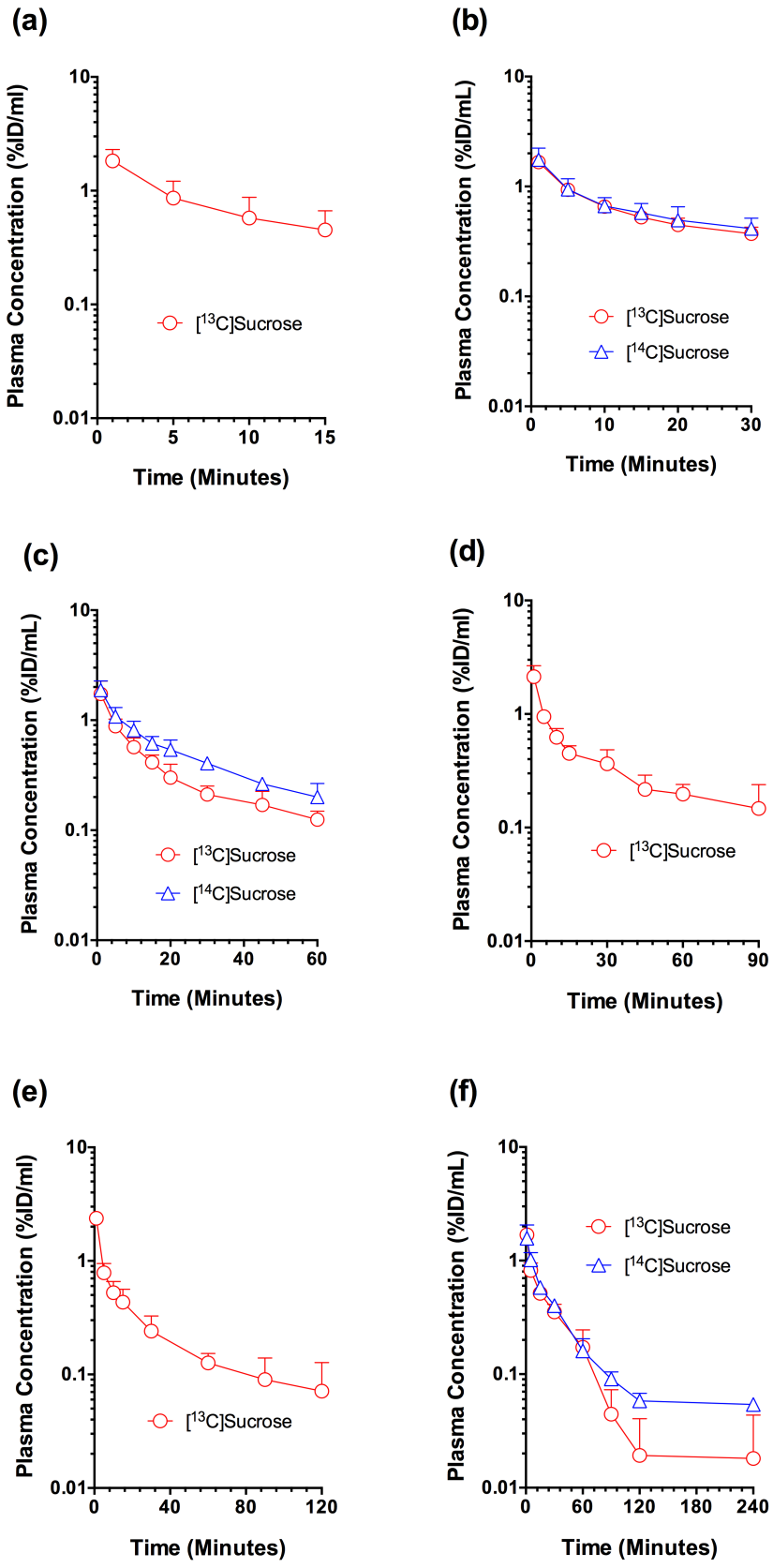

(a)
Plasma Concentration (%ID/ml)
10
1
0.1
0.01
0
5
10
15
Time (Minutes)
[13C]Sucrose
(b)
Plasma Concentration (%ID/mL)
10
1
0.1
0.01
0
10
20
30
Time (Minutes)
[13C]Sucrose
[14C]Sucrose
(c)
Plasma Concentration (%ID/mL)
10
1
0.1
0.01
0
20
40
60
Time (Minutes)
[13C]Sucrose
[14C]Sucrose
(d)
Plasma Concentration (%ID/ml)
10
1
0.1
0.01
0
30
60
90
Time (Minutes)
[13C]Sucrose
(e)
Plasma Concentration (%ID/ml)
10
1
0.1
0.01
0
40
80
120
Time (Minutes)
[13C]Sucrose
(f)
Plasma Concentration (%ID/mL)
10
1
0.1
0.01
0
60
120
180
240
Time (Minutes)
[13C]Sucrose
[14C]Sucrose

Figure 2

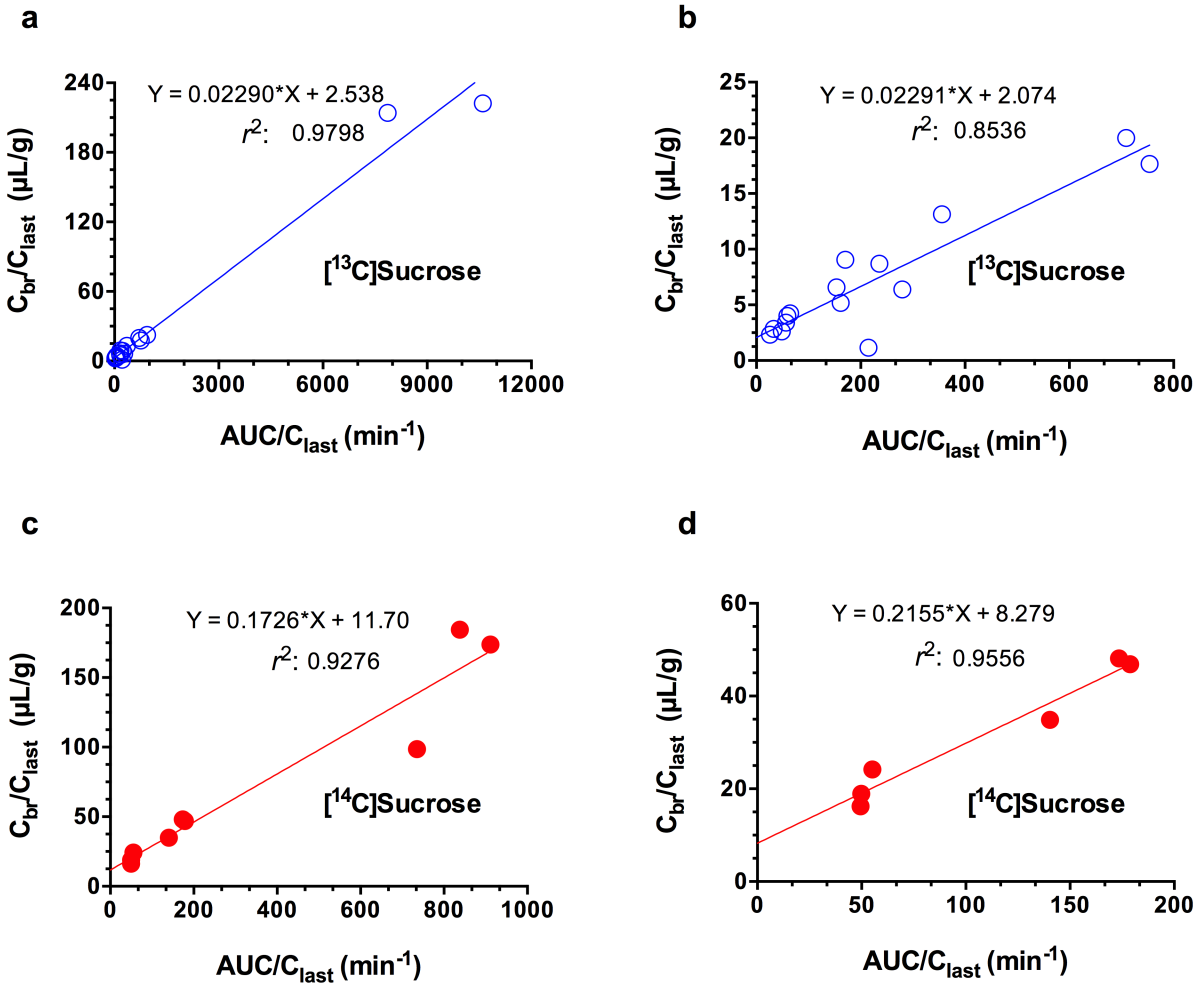

a
Y = 0.02290*X + 2.538
r2: 0.9798
[13C]Sucrose
Cbr/Clast (µL/g)
AUC/Clast (min-1)
b
Y = 0.02291*X + 2.074
r2: 0.8536
[13C]Sucrose
Cbr/Clast (µL/g)
AUC/Clast (min-1)
c
Y = 0.1726*X + 11.70
r2: 0.9276
[14C]Sucrose
Cbr/Clast (µL/g)
AUC/Clast (min-1)
d
Y = 0.2155*X + 8.279
r2: 0.9556
[14C]Sucrose
Cbr/Clast (µL/g)
AUC/Clast (min-1)

Figure 3

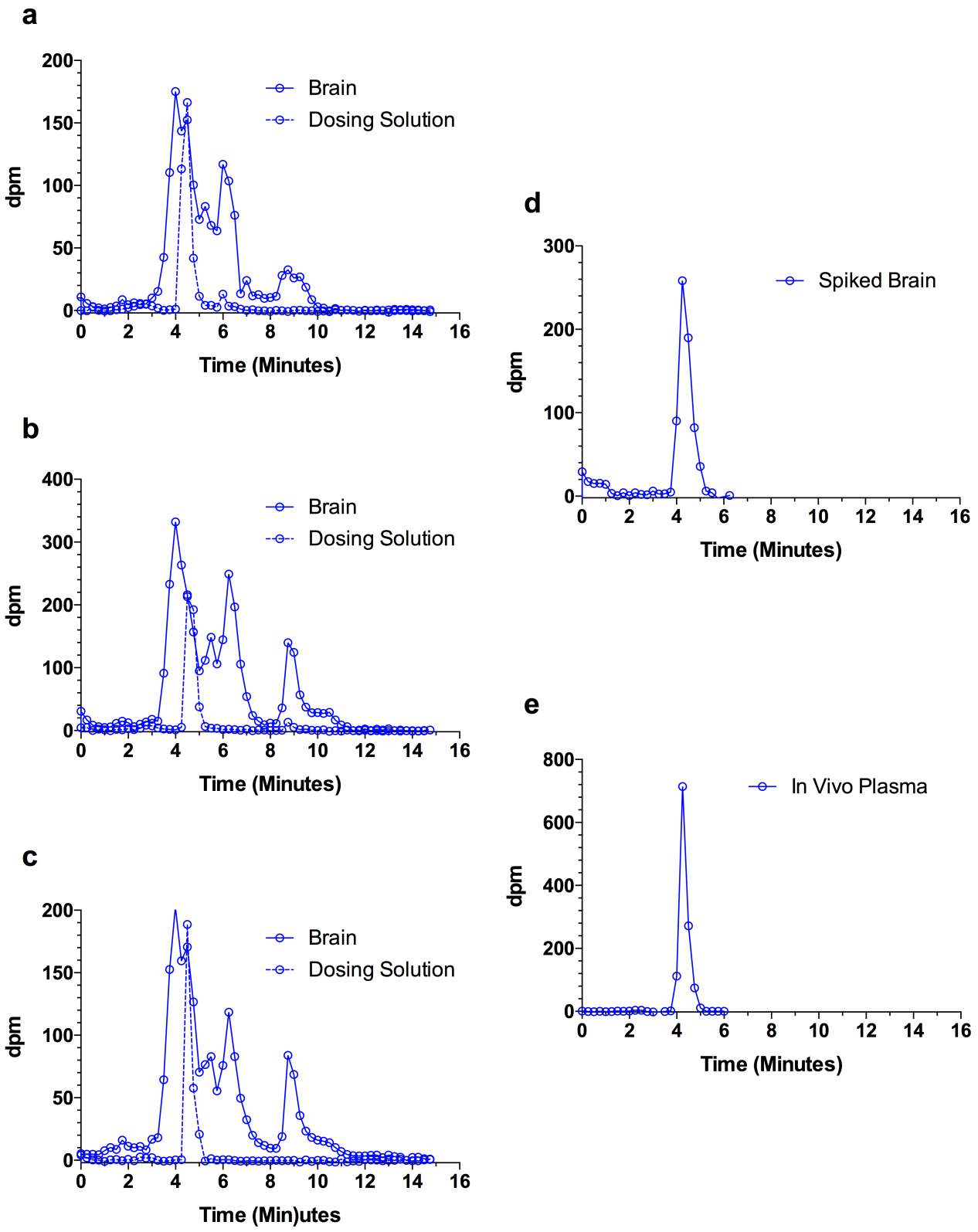

a
Brain
Dosing Solution
dpm
Time (Minutes)
b
Brain
Dosing Solution
dpm
Time (Minutes)
c
Brain
Dosing Solution
dpm
Time (Min)utes
d
Spiked Brain
dpm
Time (Minutes)
e
In Vivo Plasma
dpm
Time (Minutes)

Figure 4

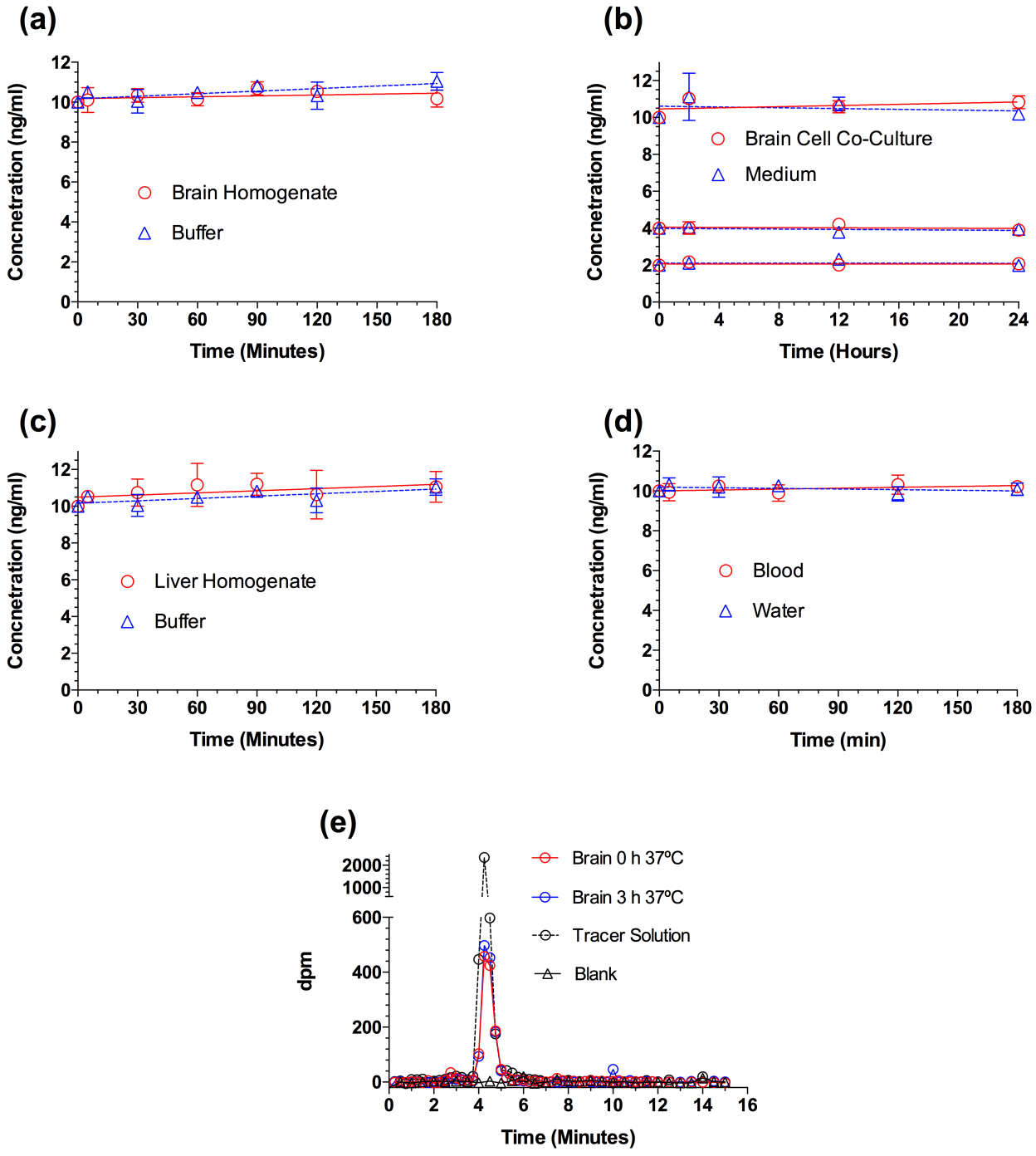
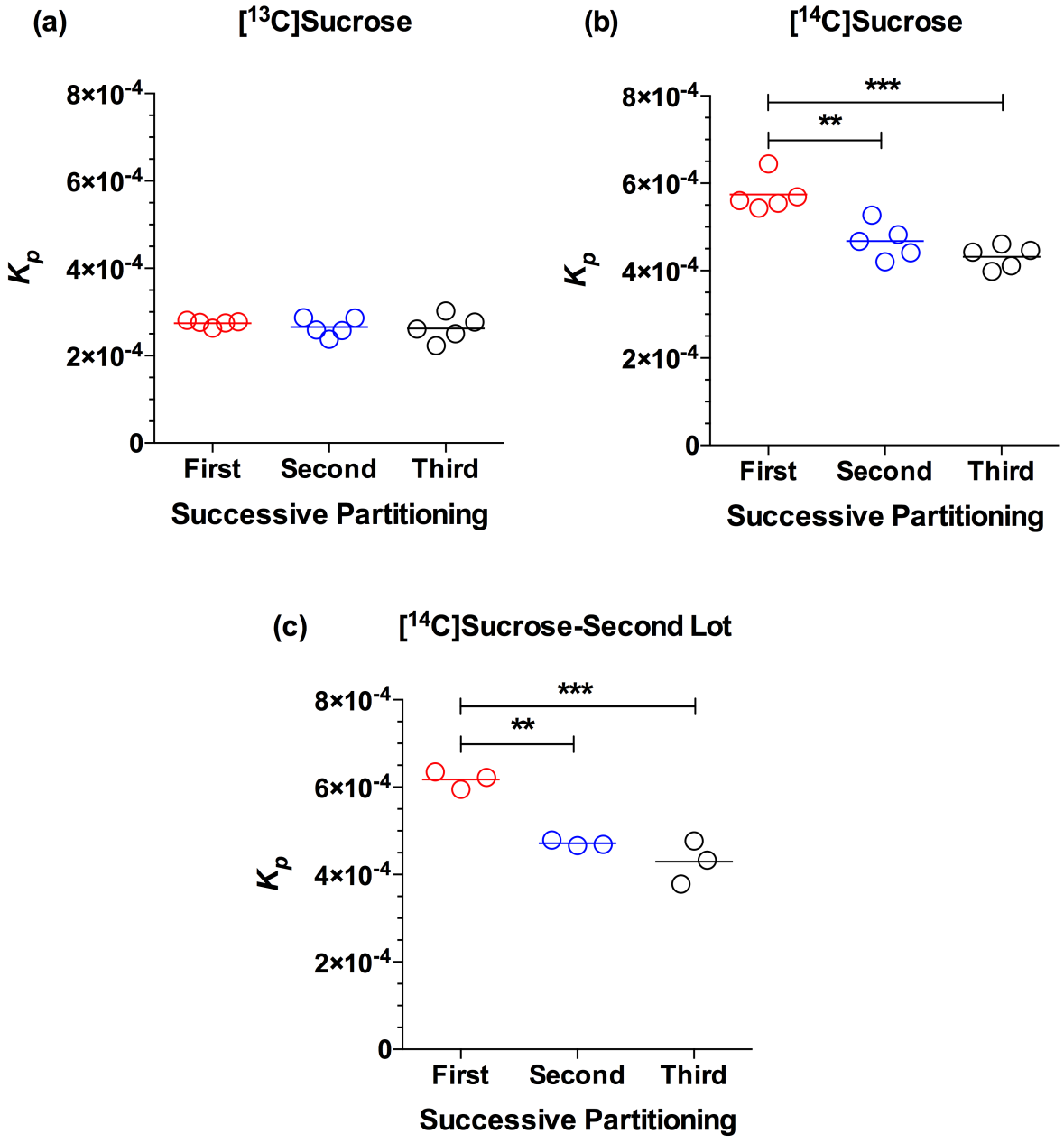

(a)
Concnetration (ng/ml)
Brain Homogenate
Buffer
Time (Minutes)
(b)
Concnetration (ng/ml)
Brain Cell Co-Culture
Medium
Time (Hours)
(c)
Concnetration (ng/ml)
Liver Homogenate
Buffer
Time (Minutes)
(d)
Concnetration (ng/ml)
Blood
Water
Time (min)
(e)
dpm
Brain 0 h 37ºC
Brain 3 h 37ºC
Tracer Solution
Blank
Time (Minutes)

Figure 5


(a)
[13C]Sucrose
8×10-4
6×10-4
4×10-4
2×10-4
0
Kp
First
Second
Third
Successive Partitioning
(b)
[14C]Sucrose
***
**
8×10-4
6×10-4
4×10-4
2×10-4
0
Kp
First
Second
Third
Successive Partitioning
(c)
[14C]Sucrose-Second Lot
***
**
8×10-4
6×10-4
4×10-4
2×10-4
0
Kp
First
Second
Third
Successive Partitioning