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Short Communication

Brain Uptake of [^{13}C] and [^{14}C]Sucrose Quantified by Microdialysis and Whole Tissue Analysis in Mice[S]

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ABSTRACT

Among small, hydrophilic drug-like molecules, [^{14}C]sucrose has long been considered the gold standard for determination of blood-brain barrier permeability. However, we have recently shown in rats that, compared with liquid chromatography–tandem mass spectrometry analysis of stable isotope (^{13}C) of sucrose, [^{14}C]sucrose significantly overestimates the brain tissue concentration and uptake of sucrose by a factor of 6 to 7. This discrepancy is due to the presence of small quantities of lipophilic impurities in [^{14}C]sucrose tracer solutions. Here, we used intracranial microdialysis to measure concentrations of both sucrose variants in brain extracellular fluid (ECF) after intravenous bolus administration to mice. Both markers displayed similar plasma profiles and ECF dialysate concentrations. However, total brain tissue concentrations and apparent brain uptake clearance of [^{14}C]sucrose were 4.1- and 3.6-fold higher, respectively, than those of [^{13}C]sucrose. Therefore, the contaminants of [^{14}C]sucrose with higher permeability were likely sequestered by brain cells, which renders them nondialyzable. It is concluded that although measurement of radioactivity overestimates the concentrations of intact sucrose in the brain tissue, the ECF radioactivity after microdialysis is a relatively accurate reflection of intact sucrose after the systemic administration of the [^{14}C]sucrose marker.

Introduction

The blood-brain barrier (BBB) represents a tight endothelial barrier protecting the central nervous system by controlled influx of substances from the circulation. The integrity of the BBB is compromised in a wide range of diseases such as stroke, multiple sclerosis, Alzheimer's disease, and brain cancer (Liebner et al., 2018). Therefore, BBB permeability is a key parameter that needs to be analyzed frequently in preclinical models. In small animals, functional damage to the BBB is typically assessed by brain tissue sampling after systemic injection of a marker that penetrates the healthy barrier poorly. Exact quantitative pharmacokinetic evaluation of passive tissue uptake is greatly facilitated when the analyte is metabolically stable in circulation, shows no significant binding to plasma or tissue proteins, is not a substrate of any of the multiple influx or efflux transport systems present at the BBB, and is nontoxic or at least does not affect the integrity of the BBB during the experiment. Experience shows that few small molecular weight chemicals match these criteria. For example, atenolol has long been considered a good model of hydrophilic drugs for measuring passive permeability across the intestinal epithelium and the BBB (Hammarlund-Udenaes et al., 1997). However, recent work by the same group revealed that, in fact, the low brain uptake of atenolol is not primarily a consequence of poor membrane permeability, but is due to substantial active efflux transport (Chen et al., 2017).

The disaccharide sucrose comes close to being an ideal small molecule marker because, after systemic administration, it is not metabolized or bound to proteins. Additionally, to date, there is no evidence that sucrose is the substrate of facilitative or active transport at the mammalian BBB. While members of the SLC45 family of proton-coupled transporters have recently been implicated as putative sucrose transporters under special conditions in mammalian tissues, including kidney and testis (Bartölke et al., 2014; Vitavska et al., 2016; Vitavska and Wieczorek, 2017), these transport proteins have higher affinity to monosaccharides, in particular for glucose and fructose (Bartölke et al., 2014). However, relevant expression levels or physiologic functions at the BBB have not been found for any of these transporters. Moreover, transport of sucrose would likely be blunted by physiologic blood glucose levels. Therefore, sucrose has been used in the BBB field for decades in the form of internally labeled radioactive tracers, most frequently as [^{14}C]sucrose. However, we have recently demonstrated that measurement of brain uptake of [^{14}C]sucrose, based on its plasma and total brain concentrations, is compromised by the presence of low quantities of lipophilic, BBB permeable impurities, which make up 80% or more of the total radioactivity in brain after intravenous administration (Miah et al., 2017b). Based on direct comparison, we suggested the use of nonradioactive [^{13}C]sucrose and ultra-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis as a better alternative (Miah et al., 2016, 2017a,b).

Intracranial microdialysis is an excellent experimental approach to measure in vivo concentrations of analytes in brain interstitial fluid, and [^{14}C]sucrose has been used repeatedly as a test or reference

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ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; AUC, area under the curve; BBB, blood-brain barrier; ECF, extracellular fluid; ID, injected dose; LC-MS/MS, liquid chromatography–tandem mass spectrometry.

substance in microdialysis (Dykstra et al., 1992; Terasaki et al., 1992; Morgan et al., 1996; Groothuis et al., 1998; Diserbo et al., 2002). We wondered if the data obtained in such experiments could also be biased by the radioactive impurities. The present study was designed to address that question by comparing brain uptake of [^{14}C] and [^{13}C]sucrose in two groups of mice after intravenous bolus administration of the markers. The comparison was based on measurement of [^{14}C] and [^{13}C]sucrose in plasma, whole brain tissue, and brain dialysate samples.

Materials and Methods

Chemicals and Reagents. [$^{13}C_{12}$]Sucrose (all 12 carbons labeled with ^{13}C) and [$^{13}C_6$]sucrose (only the six carbons in the fructose moiety labeled) were purchased from Omicron Biochemicals (South Bend, IN). [^{14}C(U)]Sucrose (uniformly labeled, specific activity 435 mCi/mmol) was supplied by PerkinElmer (Boston, MA). LC-MS/MS-grade solvents and analytical grade chemicals were obtained from reliable commercial sources.

Animals. Male C57BL/6J mice, aged 8–12 weeks, weight 25–30 g, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in ventilated cages in a temperature- and humidity-controlled room with 12-hour dark-light cycles and free access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center and complied with the National Research Council guidelines for care and use of animals (National Research Council, 2011).

LC-MS/MS Analysis of [$^{13}C_{12}$]Sucrose in Plasma, Dialysate, and Brain Samples. The permeability marker [$^{13}C_{12}$]sucrose was quantified by LC-MS/MS according to our previously published protocol using [$^{13}C_6$]sucrose as the internal standard (Miah et al., 2016). The sample preparation methods for plasma and brain homogenate were carried out according to the published method. Before analysis, microdialysate samples were diluted 1:9 in acetonitrile: water (80:20) containing 2 ng/ml internal standard. Standard curves were prepared in blank plasma (10–1000 ng/ml), blank brain homogenate (1–200 ng/ml), and artificial cerebrospinal fluid (aCSF) (1–300 ng/ml). We validated [$^{13}C_{12}$]sucrose analysis in an aCSF/brain dialysate matrix. The percent recovery ($\pm$S.D.) from aCSF samples at 10 ng/ml was 93.5 $\pm$ 10.4 and 100 $\pm$ 3 for [^{13}C]sucrose and [$^{13}C_6$]sucrose (internal standard), respectively. Inter- and intrarun accuracy was within 94%–103% for all tested concentrations in the aCSF matrix, and sample precision values (relative S.D.) were $\leq$9% for all concentrations within the range of the standard curve (1–300 ng/ml). The r^2 of the linear regression analysis of standards was $\geq$0.99.

Liquid Scintillation Counting. All radioactive samples were measured in a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Brea, CA) with appropriate window settings for ^{14}C. Quench monitoring (the H-number) and automatic quench correction were applied to convert counts per minute into disintegrations per minute. While aCSF and dialysate samples were measured in 5 ml of ScintiSafe 30% (Thermo Fisher Scientific, Waltham, MA), plasma and brain samples were first solubilized in 1 ml of Soluene350 at 65°C overnight before adding 15 ml Hionic-Fluor scintillation fluid (both obtained from Perkin Elmer, Waltham, MA).

Microdialysis Probe Construction and In Vitro Recovery of [^{13}C] and [^{14}C]Sucrose. Y-shaped self-built dialysis probes with a 10 kDa molecular weight cutoff, 280 μm o.d., and 2 mm exchange length were constructed as previously described (Sumbria et al., 2011). Random samples of probes (n = 4) from each batch were selected to test the in vitro recovery rate of the probes. The probes were immersed in a 10-ml beaker containing 1 μg/ml [$^{13}C_{12}$]sucrose or 0.5 μCi/ml [^{14}C]sucrose dissolved in aCSF that was well stirred and maintained at 37°C. Before dialysis, two 30-μl ([$^{13}C_{12}$]sucrose) or 50-μl ([^{14}C]sucrose) aliquots of the solution in the beaker were taken and 20-μl aliquots were used for analysis of the reference concentration (C_e). Next, the probes were perfused with aCSF at a flow rate of 2 μl/min, and then 20 μl dialysate samples were collected at intervals of 10 minutes over a period of 60 minutes for measurement of the outflow concentration (C_{out}). In vitro recovery in the dialysate, $R_{(\text{in vitro})}$, was calculated from the C_{out} / C_e ratio, where C_e is the average of duplicate measurements.

Microdialysis Probe Implantation and In Vivo Recovery of [^{13}C]Sucrose. Mice (n = 4) were anesthetized with isoflurane (4.0% for induction, then 1%–1.5% for maintenance) in 70% N_2O/30%O_2 using a facemask and a SurgiVet vaporizer (Smiths Medical, Norwell, MA). The head was fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and the probe was implanted into the striatum exactly as described previously (Sumbria et al., 2011). After a 24-hour recovery period, the mice were reanesthetized with isoflurane, and the implanted probe was equilibrated with aCSF for 60 minutes under a flow rate of 2 μl/min. To determine the in vivo recovery of [^{13}C]sucrose, a retrodialysis experiment in four animals was conducted by perfusion of the randomly selected probes (n = 4) with aCSF containing 100 ng/ml of [$^{13}C_{12}$]sucrose. An aliquot of the perfusate was taken for analysis of the inlet concentration, C_{in}. Six dialysis samples were collected at 15-minute intervals over 90 minutes to measure C_{out}. The in vivo recovery, $R_{(\text{in vivo})}$, which is equivalent to loss upon retrodialysis, was calculated as $R_{\text{in vivo}} = (C_{in} - C_{out}) / C_{in}$.

Analysis of Brain Uptake of [^{13}C] and [^{14}C]Sucrose by Measurement of Concentrations in Plasma, Whole Brain, and Microdialysate. To measure brain uptake of [^{14}C]sucrose or [$^{13}C_{12}$]sucrose after intravenous administration, microdialysis probes were implanted and equilibrated with aCSF for 60 minutes as described previously. Subsequently, both jugular veins of mice were exposed. A bolus dose of 40 μCi/animal of [^{14}C]sucrose (n = 5) or 10 mg/kg of [$^{13}C_{12}$]sucrose (n = 5) in 50 μl physiologic saline was injected into one jugular vein. Sequential blood samples of 30 μl each were drawn from the contralateral vein at 1, 5, 10, 20, 30, 60, and 120 minutes after injection. Simultaneously, the brain dialysates were collected every 10 or 15 minutes for [^{14}C]sucrose or [$^{13}C_{12}$]sucrose, respectively. After the 120-minute blood sample was collected, transcardiac perfusion with phosphate-buffered saline (pH 7.4) was performed through the left ventricle of the heart at a flow rate of 2 ml/min for 10 minutes. At the beginning of vascular washout, both jugular veins were cut open to ensure free outflow of blood from the brain and to visually check that the outflowing perfusion fluid was clear of blood by the end of perfusion. Next, the forebrains were collected and cleaned of meninges, separated into hemispheres and weighed. The blood samples were centrifuged at 5000g for 10 minutes at 4°C, and plasma was separated. For the [$^{13}C_{12}$]sucrose group, plasma, brain, and dialysate samples were processed as described previously and stored at −80°C until the day of analysis by the ultra-performance LC-MS/MS method. Samples in the [^{14}C]sucrose series were processed as described previously. All samples were analyzed within 1 month of collection. Our recent study (Chowdhury et al., 2018) confirmed stability of sucrose in plasma and brain samples when stored at −80°C for at least 5 months and 1 month, respectively.

Pharmacokinetic and Statistical Analysis. To compare the data of [^{14}C]sucrose and [^{13}C]sucrose, the concentrations of the markers in plasma, brain, and aCSF were corrected for the injected dose (ID) and expressed as the percentage of ID per milliliter or gram. BBB permeability was calculated as the apparent brain uptake clearance, K_{in}, from the measured concentrations in total brain tissue after 120 minutes and the plasma concentration-time area under the curve (AUC), using the equation:

$$K_{in} = C_{br}^{120} \Big/ \text{AUC}_{\text{plasma}}^{0-120},$$

where

$$\text{AUC}_{\text{plasma}}^{0-120}$$

is the AUC for [^{14}C] or [^{13}C]sucrose from time 0 to 120 minutes, calculated by the logarithmic trapezoidal rule.

The concentration of [^{14}C] or [$^{13}C_{12}$]sucrose in brain extracellular fluid (ECF), C_{ECF}, was calculated from the measured dialysate concentration ($C_{\text{dialysate}}$) and $R_{\text{in vivo}}$:

$$C_{ECF} = C_{\text{dialysate}} \big/ R_{\text{in vivo}}.$$

The ECF concentration-time course AUC from time zero to 120 minutes, AUC_{ECF}^{0-120}, was then calculated by the linear trapezoidal method for both [^{14}C]sucrose and [$^{13}C_{12}$]sucrose.

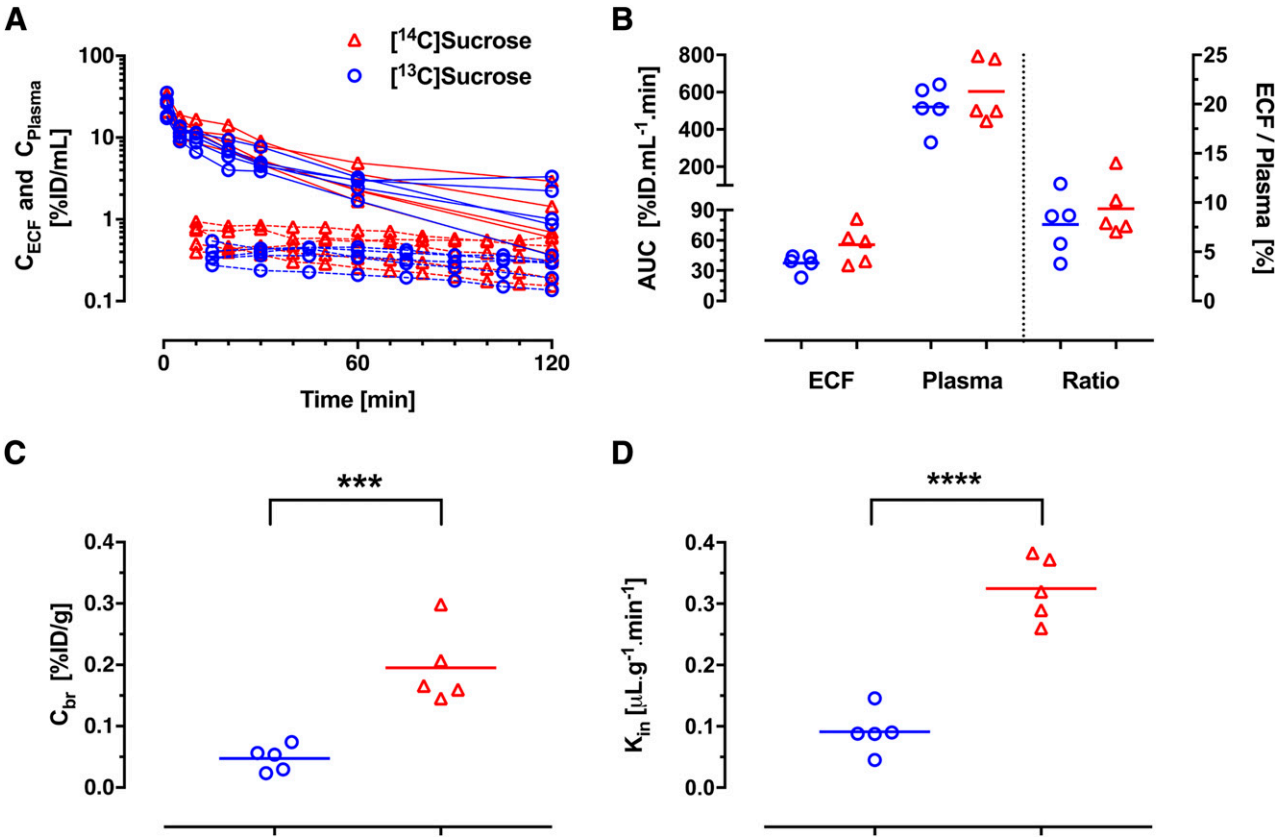

Fig. 1. The in vitro recovery rate for [^{14}C]sucrose and [^{13}C]sucrose using self-made probes with 10 kDa molecular weight cutoff ($n = 4$). Dialysate was sampled in 10-minute intervals at a flow rate of 2 μl/min. The same probes were used to determine recovery of both isotopes.

Data are presented as mean ± S.D. The kinetic parameters of [^{14}C] and [$^{13}C_{12}$]sucrose were compared by unpaired two-sided t tests. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

In Vitro and In Vivo Recovery of [^{14}C] and [^{13}C]Sucrose with Microdialysis Probes. Using a sample of the self-made probes, in vitro recovery rates for the isotopically labeled sucrose variants were not significantly different, as shown in Fig. 1, with values of 7.29% ± 0.56% and 7.03% ± 0.58% ($n = 4$) for [^{14}C] and [$^{13}C_{12}$]sucrose, respectively. Considering that the isotopic variants are chemically identical and the difference in molecular weights is minor (342.3 for [^{14}C]sucrose vs. 354.2 for [$^{13}C_{12}$]sucrose), no significant difference in probe recovery would be expected. The present values are similar to the in vitro extraction fraction of about 6% for [^{14}C]sucrose, which we found previously using probes with the same design as applied here (Bungay et al., 2011).

Consistent with experimental evidence and the theory of microdialysis in tissues, in vivo recovery rates are substantially lower than those observed in vitro due to a number of factors, including the brain extracellular space fraction of about 0.2, the tortuosity of extracellular space, and potential analyte clearance from extracellular fluid. In the current study, we determined an in vivo recovery rate of 3.88% ± 0.36% ($n = 4$) for [$^{13}C_{12}$]sucrose, which is very close to the value of 4.2% observed earlier (Sumbria, 2010) for [^{14}C]sucrose 24 hours after implantation of similar probes in mice. However, direct comparison with published data from other laboratories is limited by variations in probe geometry, molecular weight cutoff, perfusion flow rate, and animal species. In the following section, we used the in vivo recovery rate of 3.88% to calculate the in vivo concentrations of sucrose variants in brain interstitial fluid from the dialysate concentrations.

Brain Uptake of [^{14}C]Sucrose versus [$^{13}C_{12}$]Sucrose. The plasma and ECF concentration-time profiles of [^{14}C] and [$^{13}C_{12}$]sucrose are presented in Fig. 2A. Individual concentration-time courses of both variants were overlapping for both plasma and ECF samples. The AUC values (AUC_{plasma} and AUC_{ECF}) and the ratio of AUC_{ECF} to AUC_{plasma} calculated for each animal are presented in Fig. 2B. Statistical analysis revealed no significant differences between the [^{14}C]sucrose and [$^{13}C_{12}$]sucrose groups (n = 5/group) for either AUC_{plasma} (604% ± 169%ID.min.ml^{-1} vs. 521% ± 121%ID.min.ml^{-1}), AUC_{ECF} (55.6% ± 18.7%ID.min.ml^{-1} vs. 37.7% ± 8.7%ID.min.ml^{-1}) or the AUC ratios (9.37 ± 2.88 vs. 7.76 ± 3.10). Regarding the plasma concentrations, this finding is in agreement with our previous study in rats (Miah et al., 2017b), which showed no significant difference between [^{14}C]sucrose and [$^{13}C_{12}$]sucrose in terms of plasma profiles and AUC_{plasma} values. The terminal total brain tissue concentration (C_{br}^{120}) and the apparent brain uptake clearance (K_{in}) values of [^{14}C]sucrose and [$^{13}C_{12}$]sucrose are depicted in Fig. 2, C and D, respectively. The average concentration in total brain tissue of the [^{14}C]sucrose group (0.195% ± 0.062%ID/g) was 4.1-fold higher ($P = 0.0010$) than that in the [$^{13}C_{12}$]sucrose group (0.0474% ± 0.0208%ID/g), and the corresponding K_{in} value (μl.min^{-1}·g^{-1}) was 3.6-fold higher for [^{14}C]sucrose (0.325 ± 0.052 vs. 0.0913 ± 0.0587; $P < 0.0001$). These relations between the isotopic variants are similar to the data we reported previously in total brain tissue in the rat (Miah et al., 2017b), and they confirm the statement that [^{14}C]sucrose substantially overestimates brain uptake of sucrose when brain tissue is used as the source for analysis. However, the comparable concentration-time courses and AUC values in brain ECF for both isotopes

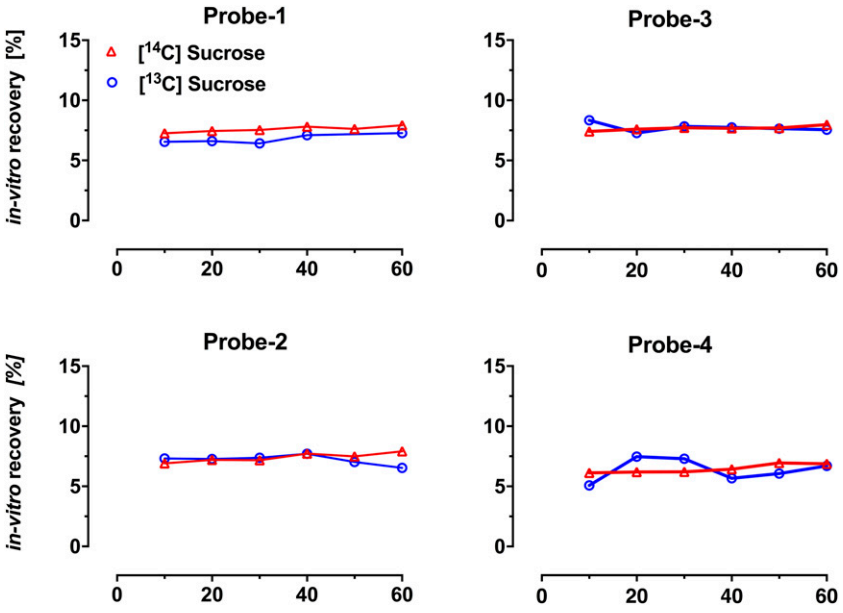

Fig. 2. (A) Plasma (C_{Plasma}, solid lines) and ECF (C_{ECF}, dashed lines) concentration profiles for [^{13}C] and [^{14}C]sucrose from 0 to120 minutes after intravenous bolus injection; (B) AUC from 0 to 120 minutes in ECF and plasma and the ECF/plasma AUC ratio of [^{13}C] and [^{14}C]sucrose; (C) terminal total brain tissue concentration (C_{br}) of [^{13}C] and [^{14}C]sucrose at 120 minutes; (D) apparent brain uptake clearance (K_{in}) of [^{13}C] and [^{14}C]sucrose over 120 minutes. Data are presented for individual animals (n = 5 for each marker). Additionally, mean values are presented as vertical bars in (B–D). ***$P < 0.001$; ****$P < 0.0001$: Significantly different based on unpaired two-tailed t test.

suggest that the fraction of ^{14}C radioactivity, which is present in ECF, behaves similar to [$^{13}C_{12}$]sucrose and can be sampled by the microdialysis technique. In contrast, the larger fraction of ^{14}C radioactivity, which accounts for the higher tissue concentrations of that tracer, must consist of nondialyzable material.

Our recent work (Miah et al., 2017b) and previous studies by others (Preston et al., 1998) suggest that the overestimations of brain concentrations and K_{in} values of [^{14}C]sucrose are due to the presence of minor lipophilic impurities in the stock solution of the marker, which easily penetrate the brain. Once beyond the brain endothelial cells and in brain interstitial fluid, the lipophilic substances will rapidly enter the intracellular compartment of brain cells (neurons, astroglia, microglia, and oligodendroglia) and are no longer available for microdialysis sampling. However, the intact sucrose is expected to be confined to the brain interstitial fluid. Indeed, the observed total brain tissue concentration of [$^{13}C_{12}$]sucrose (0.0454% ± 0.0295%ID/g) was equal to 18% of that in the ECF (0.250% ± 0.123%ID/g) at 120 minutes, which is in agreement with a brain extracellular space of 20% (Nicholson and Syková, 1998). This suggests no uptake of intact sucrose into brain cells during the 2-hour experimental period. However, the brain tissue radioactivity after [^{14}C]sucrose administration (0.195% ± 0.062%ID/g) was equal to 55% of its ECF concentration (0.354% ± 0.198%ID/g) at 120 minutes, which is much higher than the expected 20%, suggesting intracellular distribution of the radioactivity.

Although the manufacturer's data for [^{14}C]sucrose state the stock solutions are >97% pure (based on high-performance liquid chromatography), even minor lipophilic impurities that easily cross the BBB can significantly distort the brain concentrations of the intact marker because of the nonspecificity of the total radioactivity measurements. Preston et al. (1998) reported that these minor impurities, which amounted to ~2% of total activity in tracer stock solutions of [^{14}C]sucrose, were barely detectable by thin-layer chromatography. Here, we performed high-performance liquid chromatography fractionation of the [^{14}C]sucrose stock solution on a column suitable for carbohydrates. The chromatogram, shown in Supplemental Fig. 1, confirmed the radiochemical purity provided by the manufacturer's certificate of analysis. The injected dose of radioactivity was quantitatively recovered in the collected fractions. However, while a plot of the chromatogram at full scale does not suggest the presence of impurities, plotting at a 100-fold reduced scale reveals multiple small peaks of radioactivity, which cumulatively amounted to 1.98% and 0.87% of total activity eluting in fractions before and after the peak representing intact [^{14}C]sucrose, respectively (Supplemental Fig. 1). We did not attempt to identify the chemical nature of the peaks, but it is known that earlier retention times on this type of chromatographic system indicate more lipophilic compounds.

Although similar lipophilic impurities could also be present in the [$^{13}C_{12}$]sucrose stock solutions, their potential presence does not impact estimation of the brain concentration or K_{in} of the marker because of the specificity of the analytical method (LC-MS/MS), which does not measure the impurities. On the other hand, applying high-performance liquid chromatography fractionation before scintillation counting, as described previously for tracer stock, to each brain sample for measurement of intact [^{14}C]sucrose is not feasible because it would require the administration of at least 10-fold higher doses in the in vivo experiments, rendering it cost and time prohibitive.

The overestimation of brain levels of [^{14}C]sucrose due to lipophilic impurities could lead to erroneous conclusions (Miah et al., 2017a). Using a hepatic ischemia-reperfusion injury model, we demonstrated a significant increase in the K_{in} of [^{14}C]sucrose in injured rats when compared with sham-operated animals. Based on these data, it could have been concluded that hepatic ischemia-reperfusion causes damage to the BBB. However, no significant difference in K_{in} was detected in the same disease model when [^{13}C]sucrose was used as the BBB permeability marker.

In conclusion, utilizing the brain microdialysis technique in parallel to total brain tissue sampling for the comparison of two sucrose isotopes, [^{14}C]sucrose and [$^{13}C_{12}$]sucrose, as BBB permeability markers, we have shown that both isotopic variants accumulate in brain extracellular space to a similar extent after intravenous administration. However, this contrasts with the total brain tissue concentrations, which are significantly higher after systemic [^{14}C]sucrose injection. This discrepancy is likely due to the presence of minor, but highly lipophilic, impurities in the radiotracer solution. It is concluded that although measurement of radioactivity overestimates the concentrations of intact sucrose in the brain tissue, the ECF radioactivity after microdialysis is a relatively accurate reflection of intact sucrose after the systemic administration of the [^{14}C]sucrose marker.

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Performed data analysis: Alqahtani, Chowdhury, Bhattacharya, Mehvar, Bickel.

Wrote or contributed to the writing of the manuscript: Alqahtani, Mehvar, Bickel.

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