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ABSTRACT

Blood Brain Barrier (BBB) permeability is frequently compromised in the course of diseases affecting the central nervous system (CNS). Sucrose is a low molecular weight, hydrophilic marker with low permeability at the naive BBB and therefore one of the widely used indicators of barrier integrity. Our laboratory recently developed a highly sensitive UPLC-MS/MS method for stable isotope labelled [$^{13}C_{12}$]sucrose in biological matrices. Correction of total brain concentration for contribution of intravascular space is required in such experiments in order to accurately measure BBB permeability, and it is often accomplished by vascular perfusion with buffer solutions prior to brain sampling. The purpose of the present study was to develop a UPLC-MS/MS method, which allows simultaneous analysis of two different stable isotope labeled sucrose variants, one of which can be utilized as a vascular marker. The first analyte, [$^{13}C_{12}$]sucrose, serves to quantify brain uptake clearance as a measure of BBB permeability, while the second analyte, [$^{13}C_6$]sucrose, is administered just before termination of the animal experiment and is considered as the vascular marker. [$^{2}H_2$]sucrose is used as the internal standard for both ^{13}C labeled compounds. Because the majority of recent studies on CNS diseases employ mice, another objective was to validate the new technique in this species. The UPLC-MS/MS method was linear ($r^2 \geq 0.99$) in the tested concentration ranges, from 10 to 1,000 ng/mL for both analytes in plasma, from 2 to 400 ng/g [$^{13}C_{12}$]sucrose in brain and from 10 to 400 ng/g [$^{13}C_6$]sucrose in brain. It was also validated in terms of acceptable intra and inter run accuracy and precision values (n=5). The dual analyte technique was applied in a study in mice. One group received intravenous bolus injections of 10 mg/kg [$^{13}C_{12}$]sucrose at time 0, and 10 mg/kg [$^{13}C_6$]sucrose at 14.5 min, and subsequent terminal blood and brain sampling was

performed at 15 min. For comparison, another group received an intravenous bolus dose of 10 mg/kg [$^{13}C_{12}$]sucrose and was submitted to transcardiac perfusion with buffer after 15 min. We demonstrate that the two alternative techniques to correct for intravascular content deliver equivalent values for brain concentration and brain uptake clearance.

Keywords: Blood-brain barrier; Vascular space correction; Pharmacokinetics; Stable isotope labeled sucrose

1. Introduction

The microenvironment within the central nervous system (CNS) needs to be tightly controlled for optimal function, because the composition of CNS extracellular fluid (also known as cerebrospinal fluid) is unique when compared to other tissues. The precise control of substances entering or leaving the CNS is maintained by the blood-brain barrier (BBB) [1, 2]. With the help of complex intercellular tight junctions between the endothelial cells, the BBB effectively restricts the passage of ions and polar compounds, resulting in high transendothelial electrical resistance and low paracellular permeability [3]. Impairment of the BBB is either the cause or a major pathophysiological consequence of many diseases affecting the CNS (i.e., ischemic stroke, neuroinflammatory and neurodegenerative disorders). Therefore, BBB permeability is considered one of the important parameters to be monitored in experimental and clinical studies of CNS disorders [4-6].

A range of BBB permeability markers is currently in use in experimental animal models, including Evans Blue, sodium fluorescein, horseradish peroxidase, sucrose, and inulin [7-9]. Among the drug-like low molecular weight agents, radiolabeled sucrose (e.g. [^{14}C]sucrose) has long been considered the most accurate marker, explaining its frequent use [9]. We have recently introduced [$^{13}C_{12}$]sucrose as a superior marker substance, which is non-radioactive and can be quantified by a sensitive and highly specific LC-MS/MS technique [10-12]. The [$^{13}C_{12}$]sucrose technique has previously been validated in rats in our laboratory [10]. For an accurate measurement of the concentrations of the marker in the brain tissue, the method relies on removal of the marker from the brain vasculature by a brief vascular washout by transcardiac perfusion with buffer solution before tissue sampling. However, it is difficult to

judge the completeness of vascular washout in an individual animal. Additionally, there are numerous variations in technical details how the perfusions are performed, e.g., with regard to total volume, duration, flow rate, temperature and composition of perfusion fluid, which may add to the experimental variability.

An alternative is to include a second marker in the brain uptake experiments, which is injected just prior to the terminal sampling time. Although the application of a vascular marker that is physicochemically identical to the primary analyte has been advocated in the earlier BBB literature [13], emulation of this approach is rare and only few practical applications exist to date, likely due to the experimental effort required for any given analyte to reduce the concept to practice. Go and Pratt analyzed brain uptake of sodium ions using two radioisotopes with vastly different physical half-lives (^{22}Na and ^{24}Na), which allowed discrimination of the fractions of the two isotopes present in a tissue sample [14]. In an example from the other end of the molecular weight spectrum, Poduslo's laboratory corrected the brain uptake of macromolecules by radioiodinating the same protein with either ^{125}I or ^{131}I, and one labeled species was used as a vascular marker [15].

The commercial availability of different stable isotope labeled versions of sucrose allows their simultaneous use as both the BBB permeability and brain vascular space markers. Therefore, the main purpose of this study was to develop a UPLC-MS/MS method to utilize one variant of [^{13}C]sucrose as the permeability marker, another [^{13}C]sucrose version as the vascular marker, and a third, deuterium labeled sucrose, as the internal standard. Selecting suitable combinations of mass transitions and settings of the mass detector allows simultaneous LC-MS/MS quantification of the three sucrose variants, which co-elute at the identical retention

time from a BEH-amide UPLC column. Because mice are currently the most frequently used rodent model for studying the BBB, a secondary objective of our study was to validate the technique for use in mice, as opposed to our previous method for the use of [^{13}C]sucrose in rats. Applying this approach in mice, we show that the brain concentration and permeability values of sucrose can be corrected equally well as achieved by buffer washout.

2. Materials and methods

2.1. Chemicals and reagents

[$^{13}C_{12}$]Sucrose (with all 12 carbons labeled with ^{13}C isotope), [$^{13}C_6$]sucrose (with 6 of the carbons in the fructose moiety labeled with ^{13}C Isotope) and [$^{2}H_2$]sucrose ([6,6'-$^{2}H_2$ fru]sucrose) were purchased from Omicron Biochemicals (South Hill Street South Bend, IN, USA). Analytical grade ammonium hydroxide, LC–MS/MS grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). For anesthesia, isoflurane was 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.

2.2. Animals

Female C57Bl/6 mice, 8-10 weeks of age, body weight 24-27 g, were supplied by Jackson Laboratories (Bar Harbor, ME, USA) and used in the pharmacokinetic study. Mice had free access to standard rodent chow and water and were housed in an AAALAC-accredited animal

facility under controlled humidity and temperature conditions with a 12h light/12h dark cycle. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center and conformed to current NIH guidelines.

2.3. Mass spectrometric and chromatographic conditions

Analyte detection was performed using an AB SCIEX QTRAP® 5500 triple quadrupole mass spectrometer (MS) attached to a Nexera UPLC system (Shimadzu Corporation). The UPLC system contained an autosampler (Sil-30AC), pumps (LC-30AD), a controller (CBM-20A), a degasser (DGA-20A5), and a column oven (CTO-30A). Data acquisition and quantification was done with the help of Analyst software. An Acquity BEH amide column (2.1 mm x 50 mm, 1.7 µm; Waters , Milford, MA, USA) was used for chromatographic separation with isocratic elution in acetonitrile: water: ammonium hydroxide (72:28:0.1, v/v), at a flow rate of 0.2 mL/min. Column temperature was maintained at 45°C. Total run time per injection was 6 minutes.

Electrospray ionization in negative mode was used as the ionization source. Optimization of mass spectrometer parameters for [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose was already shown in our previous study [10]. The mass spectrometer conditions for [$^{2}H_2$]sucrose were optimized to get optimum $M-H^{-1}$ signal by continuous infusion of the standard solution with an infusion pump. The optimized mass spectrometer parameters were as follows: ion spray voltage, −4500 V; collision gas, high; curtain gas, 30 psi; temperature, 600°C; ion source gas 1 (nebulizer gas), 55 psi; and ion source gas 2 (turbo gas), 55 psi. For [$^{13}C_{12}$]sucrose, the m/z transition 353 → 92 was selected. For [$^{13}C_6$]sucrose, the transitions 347 → 89 and 347 → 59 are

applicable, and the latter was preferable for brain matrix (as discussed below in section 3.2). The m/z transition 343 → 89 was selected for [$^{2}H_{2}$]sucrose.

2.4. Preparation of standard curves for the dual analytes

In preparing standard curves for the dual analyte application, [$^{13}C_{12}$]sucrose and [$^{13}C_{6}$]sucrose were considered as the analytes, whereas [$^{2}H_{2}$]sucrose was used as the internal standard. For plasma standard curves, blank mouse plasma was spiked with [$^{13}C_{12}$]sucrose and [$^{13}C_{6}$]sucrose stock solutions in water to get plasma concentrations of 1-100 µg/mL. The individual plasma calibration standards were then diluted 100-fold with water to produce concentrations within the range of 10-1,000 ng/mL, before being subjected to the sample preparation procedure detailed below. In case of the brain standard curve, blank brain tissue was homogenized in water (1:9) and both analytes were spiked into the homogenate to obtain concentrations ranging from 2 to 400 ng/mL for [$^{13}C_{12}$]sucrose and 10 to 400 ng/ml for [$^{13}C_{6}$]sucrose.

2.5. Sample preparation

As mentioned above, plasma calibration standards and unknown samples were diluted 100 times with LC-MS/MS grade water, and brain samples were homogenized in water (1:9) before analysis. Deproteination for all samples was achieved in a microcentrifuge tube by diluting at a ratio of 1:9 in acetonitrile: water (80:20) containing 10 ng/mL of internal standard. Precipitated samples were vortexed, followed by centrifugation at 20,000 g for 10 minutes. The supernatant was transferred into autosampler inserts. A sample volume of 5 µL was injected onto the UPLC column.

2.6. Method validation

2.6.1. Selectivity

Selectivity of the method was ensured by running blank matrix samples containing no analyte. Also in order to ensure that there is no interference in among the analyte transitions, neat samples containing single analytes without any matrix prepared in mobile phase composition were run using the method defined above.

2.6.2. Accuracy and precision

The accuracy and precision of the method was determined through inter and intra-day runs, where quality control samples were evaluated against calibration curves. For calculation, five repeats of low, medium, and high concentrations were analyzed. The accuracy was calculated as percentage of measured concentration over nominal concentration. Precision was calculated as percentage of relative standard deviations (R.S.D.). The acceptable inter and intra-run limits for the accuracy were set at 85–115% for the middle and high concentrations and 80–120% for the low concentration. The acceptable precision values were 15% (medium and high concentrations) or 20% (low concentration) [16].

2.6.3. Linearity

The linearity of calibration curves was evaluated by the coefficient of variation (r^2) of the linear regression analysis of the concentration-response data using a weight of 1/x, where x is the concentration. Weighting by 1/x is superior to analysis with equal weights, because the latter results in greatly diminished accuracy and high variability at low concentrations.

2.6.4. Recovery

Recovery of the analytes was checked and found not to be subject to matrix effects in the previously developed method for both [$^{13}C_{12}$]sucrose as well as [$^{13}C_6$]sucrose [10]. In the dual analyte method, when [$^{2}H_2$]sucrose is considered as the new internal standard, we expected that the recovery of the analytes [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose will remain similar to the results described previously, since all of the analytes and the new internal standard are stable labelled isotopes of the same chemical entity. Nevertheless, a recovery study of the analytes was performed in all of the concerned matrices at low, medium and high-level concentrations as follows (n = 5): In case of [$^{13}C_{12}$]sucrose, the concentrations were, 100 and 1000 ng/mL for plasma matrix and 2, 50 and 400 ng/mL for brain samples. In case of [$^{13}C_6$]sucrose, the concentrations were 10, 100 and 1000 ng/mL for plasma matrix and 10, 50 and 400 ng/mL for brain samples. Five replicate samples were prepared in each of the concerned matrices, and samples with equivalent concentrations prepared in water (instead of matrix) were used as references. The samples and references were subjected to the sample preparation method described above, and the peak areas of [$^{13}C_{12}$]sucrose, [$^{13}C_6$]sucrose and the internal standard [$^{2}H_2$]sucrose were determined. Recovery was calculated as percent of the ratio of peak areas Sample/Reference, where sample refers to the matrix and reference to water (neat sample), respectively.

2.6.5. Freeze thaw stability

We subjected neat samples of the analytes at a concentration of 500 ng/mL to three freeze/thaw cycles. Samples were stored at -80°C and thawed at room temperature for one

hour, replicating experimental conditions. Concentration of the analytes in the neat samples was compared against a standard curve.

2.6.6 Long-term, storage stability of unprocessed and short-term stability of processed samples

Long term, storage stability was checked for the diluted plasma samples and brain homogenates at -80°C. Quality control samples at low, medium, and high concentrations of analytes in the brain and plasma (*n* = 3) were stored at -80°C for one month and 5 months, respectively. The stored samples were then compared against standards in order to assess stability. Similarly, for the processed sample stability, quality control samples in both matrices (*n* = 3) were subjected to the sample preparation method above, and the supernatant after protein precipitation was stored at 4°C for 24 hours before injection by the autosampler.

2.7. Application of the method

A pharmacokinetic study was conducted in two groups of anesthetized C57BL/6 mice. Isoflurane (4% for induction, 1.5-2% v/v for maintenance) in 70% nitrous oxide / 30% oxygen at a flow rate of 1 L/min was applied via a silicone face mask. The jugular veins were bilaterally exposed by skin incisions at the neck for IV injections on one side and blood sampling from the contralateral side. 10 mg/kg [$^{13}C_{12}$]sucrose in saline was injected as an IV bolus dose. Blood samples (30 µL) were collected at 0.5, 1, 2, 5, 10, and 15 min after injection to allow for analysis of the plasma concentration time curve in each individual animal. In one group of animals (Washout Group, *n* = 6), the thorax was opened immediately after the 15 min blood sample, and vascular perfusion was performed via the left ventricle of the heart at a flow rate of 2

mL/min with a total of 20 ml phosphate buffered saline (pH 7.4) at room temperature, using a Harvard syringe pump. At the start of perfusion both jugular veins were cut open to facilitate outflow of blood from the brain and to visually confirm that the outflowing perfusion fluid was clear of blood by the end of the perfusion, when brain was sampled. In the second group of animals (Vascular Marker Group, *n* = 7), an IV bolus dose of [$^{13}C_6$] sucrose (10 mg/kg in saline) was injected at 14.5 min, 30 sec prior to the terminal sampling time point. These animals were euthanized by decapitation immediately after the terminal blood sample had been collected. Blood samples were centrifuged and plasma was separated. Brains were removed from the skull, cleaned of meninges, and the forebrains (without olfactory bulbs, cerebellum or brain stem) were weighed and flash frozen in isopentane/dry ice. At the end of each experimental day, brain and plasma samples were homogenized/diluted in water as described in the sample preparation step and stored at -80°C until the day of measurement by the UPLC-MS/MS system. Brain tissue concentration values in the Washout Group, which had undergone buffer washout, were considered as corrected for intravascular content, ($C_{br-corr}^{C_{12}}$). The corresponding values of corrected brain concentrations in the Vascular Marker Group, receiving the vascular marker injection without buffer washout, were determined as follows:

$$C_{br-corr}^{C_{12}} = \frac{(V_d - V_0) \times C_{pl}^{C_{12}}}{1 - V_0} \quad (1)$$

where V_d is apparent volume of distribution of the BBB permeability marker, [$^{13}C_{12}$]sucrose, V_0 is apparent volume of distribution of the vascular marker, [$^{13}C_6$]sucrose, and $C_{pl}^{C_{12}}$ is the terminal

(15 min) plasma concentration of [$^{13}C_{12}$]sucrose. The V_d and V_0 values were estimated using the following two equations.

$$V_d = C_{br}^{C_{12}} / C_{pl}^{C_{12}} \quad (1a)$$

$$V_0 = C_{br}^{C_6} / C_{pl}^{C_6} \quad (1b)$$

where $C_{br}^{C_{12}}$ and $C_{br}^{C_6}$ are the total (uncorrected) brain concentrations of [$^{13}C_{12}$]sucrose and [$^{13}C_6$] sucrose, respectively, at the terminal sampling time (15 min), and $C_{pl}^{C_6}$ is the terminal plasma concentration of [$^{13}C_6$] sucrose at 15 min.

Values for brain uptake clearance, K_{in}, also known as the permeability-surface area product, were calculated using the following equation [17] based on either uncorrected ($C_{br}^{C_{12}}$) or corrected ($C_{br-corr}^{C_{12}}$) brain concentrations of [$^{13}C_{12}$]sucrose:

$$K_{in} = C_{br}^{C_{12}} / AUC_0^T \quad (2)$$

$$K_{in-corr} = C_{br-corr}^{C_{12}} / AUC_0^T$$

where AUC_0^T is the area under the plasma concentration time curve from time 0 to the terminal sampling time (15 min) for [$^{13}C_{12}$]sucrose. AUC was determined by the linear-logarithmic trapezoidal method using the non-compartmental analysis (NCA) module in Phoenix WinNonlin.

3. Results

3.1. MS parameter optimization of [$^{2}H_2$]sucrose

Figure 1 depicts the mass spectra with precursor and product ions for [$^{13}C_{12}$]sucrose (a), [$^{13}C_6$]sucrose (b), and [$^{2}H_2$]sucrose (c) after optimization of the MS parameters. Similar to our previous report [10], the mass spectra obtained for the 2 ^{13}C-labelled isotopes of sucrose showed m/z transitions of 353>92 and 347>89 for [$^{13}C_{12}$]sucrose (Fig. 1a) and [$^{13}C_6$]sucrose (Fig. 1b), respectively. In case of [$^{13}C_6$]sucrose, the m/z transition of 347>59 is also a viable alternative (Fig. 1b). For the newly optimized [$^{2}H_2$]sucrose, the m/z transition of 343>89 was found to be the best transition in terms of signal to noise ratio (Fig. 1c). In the previously described single analyte method, [$^{13}C_6$]sucrose was utilized as the internal standard. However, repeat injections of the same concentrations of [$^{13}C_6$]sucrose and [$^{2}H_2$]sucrose (Fig. 2) showed that [$^{13}C_6$]sucrose provided higher sensitivity (larger peak areas and signal to noise ratios) compared to [$^{2}H_2$]sucrose. As a consequence, we chose [$^{13}C_6$]sucrose as the second analyte (i.e., vascular marker) and [$^{2}H_2$]sucrose as the internal standard.

3.2 Selectivity

Single analyte neat samples prepared in water showed no cross-channel interference between transitions in the absence of matrix (Fig. 3). Chromatograms of samples containing blank, lowest standards and internal standard in mouse plasma and mouse brain are depicted in Fig. 4. The matrix samples were found to be acceptable without any interference except for transition 347>89 of [$^{13}C_6$]sucrose, which had an interfering peak in the blank brain samples at the same retention time as the analyte (approx. 2.1-2.2 min). The transition 347>59 had a peak

at retention time approx. 2.6 minute in brain matrix, which does not interfere with the analyte peak at retention time of 2.1 min, and hence was chosen for measurement of brain samples.

3.3 Accuracy and precision

The results of the inter and intra run accuracy and precision experiments in plasma and brain samples are presented in Tables 1 and 2, respectively. Accuracy and precision values of the quality control samples in low, middle and high-level QCs across both tested matrices were found to be within the acceptable limits.

3.4. Linearity

Calibration curves in both tested matrices across all intra and inter assay runs were found to be linear with r^2>0.99.

3.5. Recovery

The recoveries (mean ± SD) of [$^{13}C_{12}$] and [$^{13}C_6$]sucrose from the plasma and brain samples at all the concentrations (n = 5) were quantitative. Recoveries of [$^{13}C_{12}$]sucrose from plasma samples at nominal concentrations of 10, 100, and 1000 ng/mL were 104 ± 9, 97.5 ± 4.5, and 105 ± 8 percent, respectively. Recoveries of [$^{13}C_6$]sucrose from plasma at the same concentrations were 99.6 ± 13.6, 99.0 ± 6, and 106 ± 9 percent. For [$^{13}C_{12}$]sucrose in brain homogenate at 2, 50, and 400 ng/mL, recoveries were 94.1 ± 4.2, 102 ± 3 and 100 ± 1 percent, respectively. For [$^{13}C_6$]sucrose in brain homogenate at 10, 50, and 400 ng/mL, recoveries were 108 ± 11, 106 ± 4 and 99.2 ± 2.7 percent, respectively.

3.6. LLOD and LLOQ

As apparent from Fig. 4 and Tables 1 and 2, our method can accurately and precisely measure [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose at the lowest concentrations of the respective calibration curves (10 ng/mL in diluted plasma for both analytes, 2 ng/g brain for [$^{13}C_{12}$]sucrose and 10 ng/g for [$^{13}C_6$]sucrose). Based on a signal to noise ratio of ≥ 3, the LLOD of the assay on column is 0.1 pg, as previously reported [10]

3.7. Stability

Results from the long-term, storage stability of unprocessed and short-term stability of processed samples are shown in Table 3. Additionally, Fig. 5 depicts the freeze thaw stability data. All data were found to be within specifications. Analytes were found to be stable in plasma and brain matrix over long term (Table 3). Also, processed samples stability data suggest that the processed samples could be stored at 4^{o}C for up to 24 hours before analysis (Table 3). However, we found that at 48 hours the peak shape degraded, indicating a storage time of processed samples beyond 24 hours may not be suitable (data not shown).

3.8 Application of the method

The results of the pharmacokinetic study are depicted in Fig. 6. All concentrations of analytes [$^{13}C_{12}$]sucrose or [$^{13}C_6$]sucrose measured from brain and plasma samples fell within the range of the respective standard curves. As expected, plasma concentration – time curves in the two groups of mice were similar (Fig. 6a), and the areas under curve from 0 to 15 min were not significantly different (unpaired, two-tailed, t-test). After considering the dilution factor of 10 for brain samples, the average values (±SD) of brain concentrations (Fig. 6b) were as follows:

Brain concentration of the BBB permeability marker [$^{13}C_{12}$]sucrose in the Washout group was 129 ± 26.0 ng/g; brain concentration of [$^{13}C_{12}$]sucrose in the Vascular Marker group was 265 ± 42 ng/g before correction for intravascular content, and 138 ± 27 ng/g after correction (see **Fig. 6b**). Comparison of the corrected brain concentrations (washout vs. vascular marker correction) showed no significant difference ($p = 0.59$, unpaired, two-tailed t-test). Similarly, the values for brain uptake clearance, $K_{in\text{-}corr}$, were almost identical under both techniques: 0.257 ± 0.053 µL min^{-1} g^{-1} vs. 0.260 ± 0.047 µL min^{-1} g^{-1} for the Washout and Vascular Marker groups, respectively, as depicted in Fig 6c. Without correction for the intravascular content, the estimate of apparent K_{in} in the Vascular Marker group would be 0.501 ± 0.064 µL min^{-1} g^{-1}. The estimated intravascular plasma space, V_0, as represented by the apparent volume of distribution of the vascular marker, was 9.12 ± 1.89 µL g^{-1}.

4. Discussion

The results of this study represent an extension and refinement of the previously described method [10] for measurement of [$^{13}C_{12}$]sucrose in brain and plasma by UPLC-MS/MS. Sample preparation and quality control experiments demonstrated that measuring stable labelled isotopes of sucrose in plasma and brain matrices from mice can be performed with similar assay quality as in rats [10]. Accuracy and precision values for the lowest calibration standard (Tables 1 and 2), along with linearity of the standard curves, were all well within the defined specifications. Similar to previous findings [10, 18], the recovery from the matrices

throughout the concentration ranges covered by the standard curves was found to be almost quantitative for both [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose.

The dual analyte method with a vascular marker is particularly attractive in the case of sucrose, because several isotopically labelled variants of sucrose are readily commercially available. As they are chemically equivalent and co-elute from a suitable stationary phase, such as the BEH amide column used here, variation of detector signal due to confounding factors, like differences in ion suppression between analytes and/or internal standard, is expected to be minimal. As shown in Fig. 4, equivalent mass transitions for all three sucrose isotopes can be chosen for analysis in the various matrices, with the exception of [$^{13}C_6$]sucrose in brain. Blank brain matrix showed a peak at the same retention time as the analyte at transition m/z 347>89. Transition 347>59 m/z provided a better signal to noise ratio and was used for quantification of [$^{13}C_6$]sucrose in brain matrix. Since the in vitro stability of [$^{13}C_{12}$]sucrose in cell culture media as well as brain homogenate was tested in a previous study for up to 3 hours [12], in this study we also included processed samples stability, long term stability as well as freeze thaw stability in order to confirm that the analytes stay stable in the applied conditions for specified periods of time. Since both analytes are different stable isotope labeled versions of the same molecule, we can imply that the stability for [$^{13}C_{12}$]sucrose is also valid for [$^{13}C_6$]sucrose.

We found that for physiological applications, such as brain uptake experiments, the dual analyte technique is equally effective as buffer perfusion. As demonstrated in the mice receiving a vascular marker injection, the intravascular content, if left uncorrected, would cause significant overestimation of brain concentration and K_{in} by a factor of almost 2 in the present experiments with brain sampling at 15 min (Fig. 6b and c). At shorter terminal sampling time

points that error would be even more pronounced, because plasma concentrations are still higher (Fig. 6a), and therefore the ratio of vascular content to total brain concentration is also higher. Because the vascular marker itself is subject to brain uptake during the brief interval between injection and brain sampling, intravascular volume is slightly overestimated, and correspondingly, the calculated values of corrected brain concentration and K_{in} for the permeability marker [$^{13}C_{12}$]sucrose, would be slightly underestimated. However, as shown by our data, that error is too small to result in significant differences when compared to the vascular washout procedure. Moreover, the V_0 value of 9.12 ± 1.89 µL g^{-1}, as measured with [$^{13}C_6$]sucrose in the present study, is almost identical to the brain plasma volume of 9.3 ± 1.1 µL g^{-1} as determined by our laboratory with radiolabeled IgG in a previous study in mice [19], and it falls into the middle of the range of values reported in the literature for rodents [20].

There are distinct practical advantages of the correction method by vascular marker administration over the washout method: It is technically simpler, and collection of brain tissue is feasible within seconds after the terminal blood sampling, rather than being delayed several minutes by thoracotomy and perfusion (e.g., over 10 min in the present study). Additionally, rapid sampling is important when, in addition to measurement of BBB permeability, parts of the brain samples were to be used for measurement of other analytes that may be subject to rapid degradation, such as neurotransmitters or metabolites.

5. Conclusions

In conclusion, the previously described UPLC-MS/MS method with a single analyte has been refined by the addition of a second stable isotope labeled sucrose variant, which serves as a vascular marker. The method allows measurement of both analytes in the same sample in a single run. This technique simplifies correction for intravascular plasma space in brain uptake experiments with sucrose and makes a vascular washout step dispensable.

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Table 1

Intra- and inter-run values for accuracy and precision of lowest, middle and highest concentrations of [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose in the calibration curves in mouse plasma (n = 5).

Analyte	Added Conc. (ng/ml)	Intraday run			Interday run		
		Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)	Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)
[$^{13}C_{12}$]sucrose	10	10.1	101	2.68	9.76	97.6	3.18
	100	97.9	97.9	1.34	97.1	97.1	1.18
	1000	984	98.4	1.69	980	98.0	0.98
[$^{13}C_6$]sucrose	10	9.82	98.2	6.29	10.4	104	2.69
	100	96.0	96.0	2.27	96.2	96.2	2.18
	1000	983	98.3	3.45	986	98.6	3.38

Table 2

Intra-day and inter-day run values for accuracy and precision of lowest, middle and highest concentrations of [$^{13}C_{12}$]sucrose and [$^{13}C_6$]sucrose in the calibration curves in mouse brain homogenate (n = 5)

Analyte	Added Conc. (ng/ml)	Intraday run			Interday run		
		Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)	Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)
[$^{13}C_{12}$]sucrose	2	1.98	99.1	3.52	1.98	99.3	7.23
	50	48.3	96.6	1.08	49.5	98.9	1.84
	400	372	92.9	3.71	416	104	4.24
[$^{13}C_6$]sucrose	10	10.1	101	6.06	9.96	99.6	7.67
	50	48.2	96.3	2.72	48.8	97.6	2.25
	400	380	95.0	5.05	414	103	3.11

Table 3

Stability of [$^{13}C_{12}$]sucrose during long-term storage (5 months for plasma and 1 month for brain homogenate, -80°C) of unprocessed and short-term storage (24 hr for both plasma and brain homogenate, 4°C) of processed samples (*n* = 3).

Sample	Added Conc. (ng/ml)	Long-term storage stability of unprocessed samples			Short-term storage stability of processed samples		
		Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)	Measured Conc. (ng/ml)	Accuracy (%)	Precision (RSD %)
Plasma	10	9.68	96.8	3.22	10.0	100	1.91
	100	102	102	1.72	98.4	98.4	2.65
	1000	1000	100	2.62	1040	104	2.52
Brain homogenate	2	1.98	98.8	9.94	2.00	100	8.41
	50	49.2	98.4	2.79	48.1	96.3	1.92
	400	389	97.3	5.46	423	106	2.94

Legends for Figures

Figure 1: Mass spectra with precursor and product ions for [$^{13}C_{12}$]sucrose (a), [$^{13}C_6$] Sucrose (b), and [$^{2}H_2$]sucrose (c)

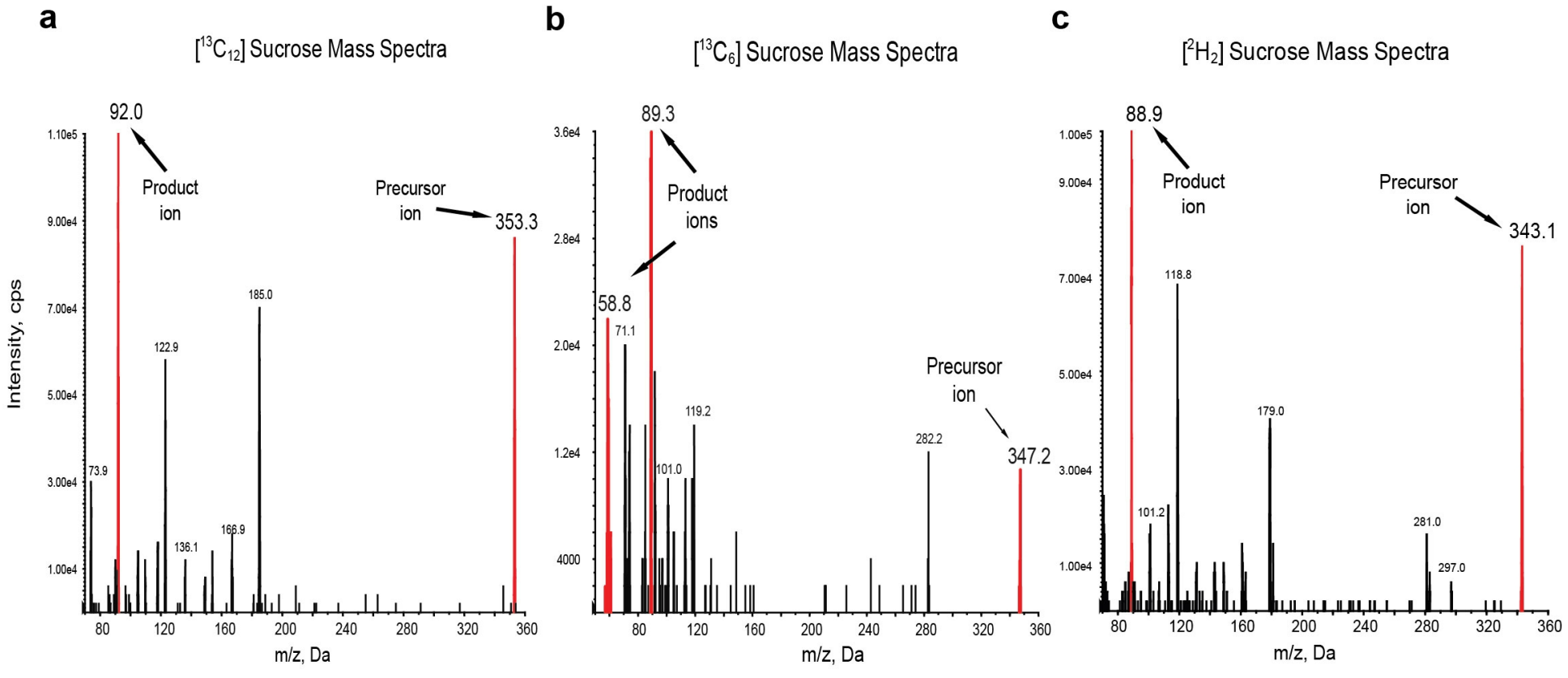
Figure 2: Comparison of peak areas of [$^{2}H_2$]sucrose and [$^{13}C_6$]sucrose obtained in repeated injections under optimized detection conditions (mean ± SD, n=15). [$^{13}C_6$]sucrose produces consistently higher peak areas and achieves higher sensitivity.

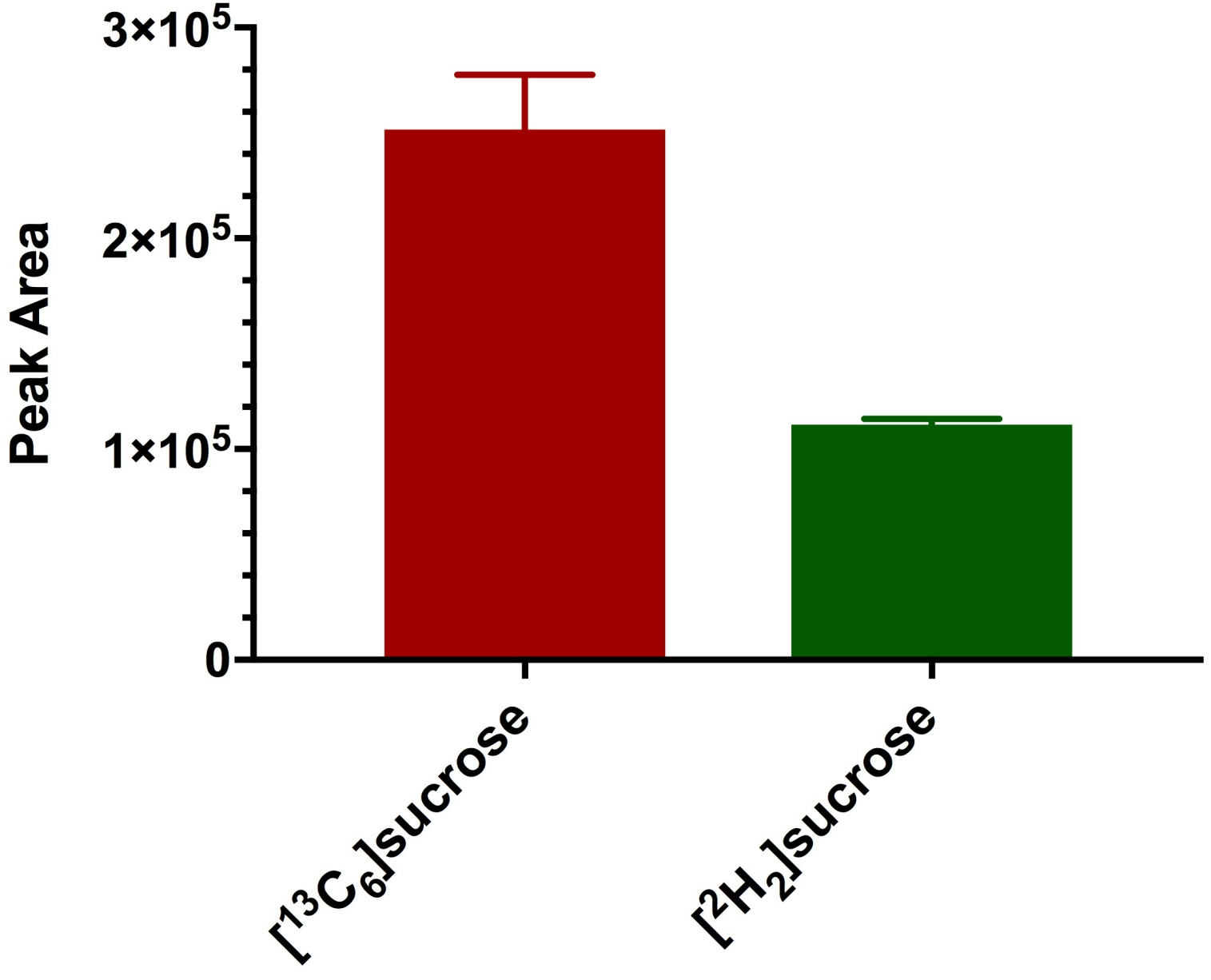
Figure 3: Chromatograms of single analyte neat samples of [$^{13}C_{12}$] Sucrose, [$^{13}C_6$] Sucrose and [$^{2}H_2$] Sucrose, prepared in LC-MS/MS grade water in all considered transitions.

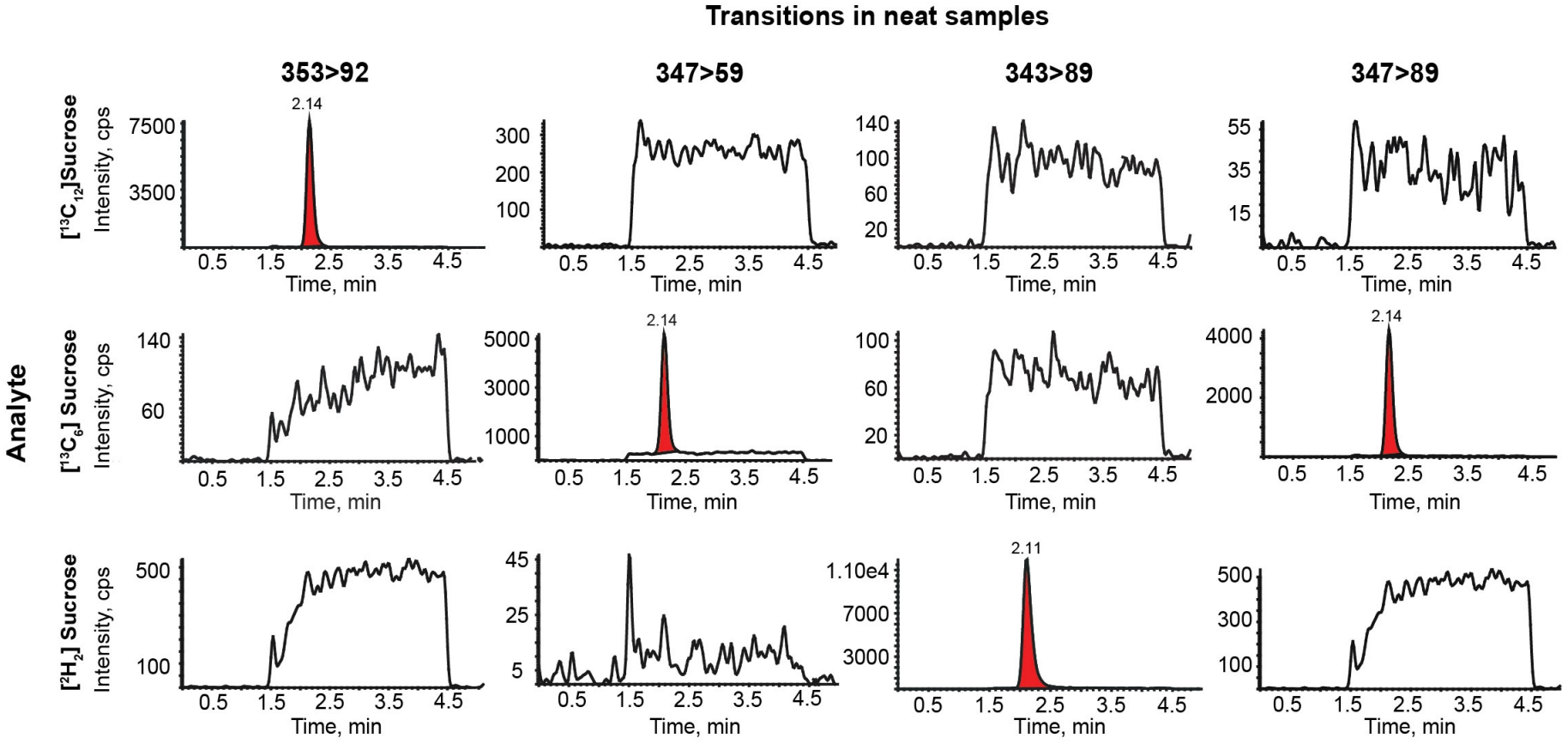
Figure 4: Chromatograms of blank matrices, lowest calibration standard and internal standard in Plasma (Panel a) and Brain Matrix (Panel b).

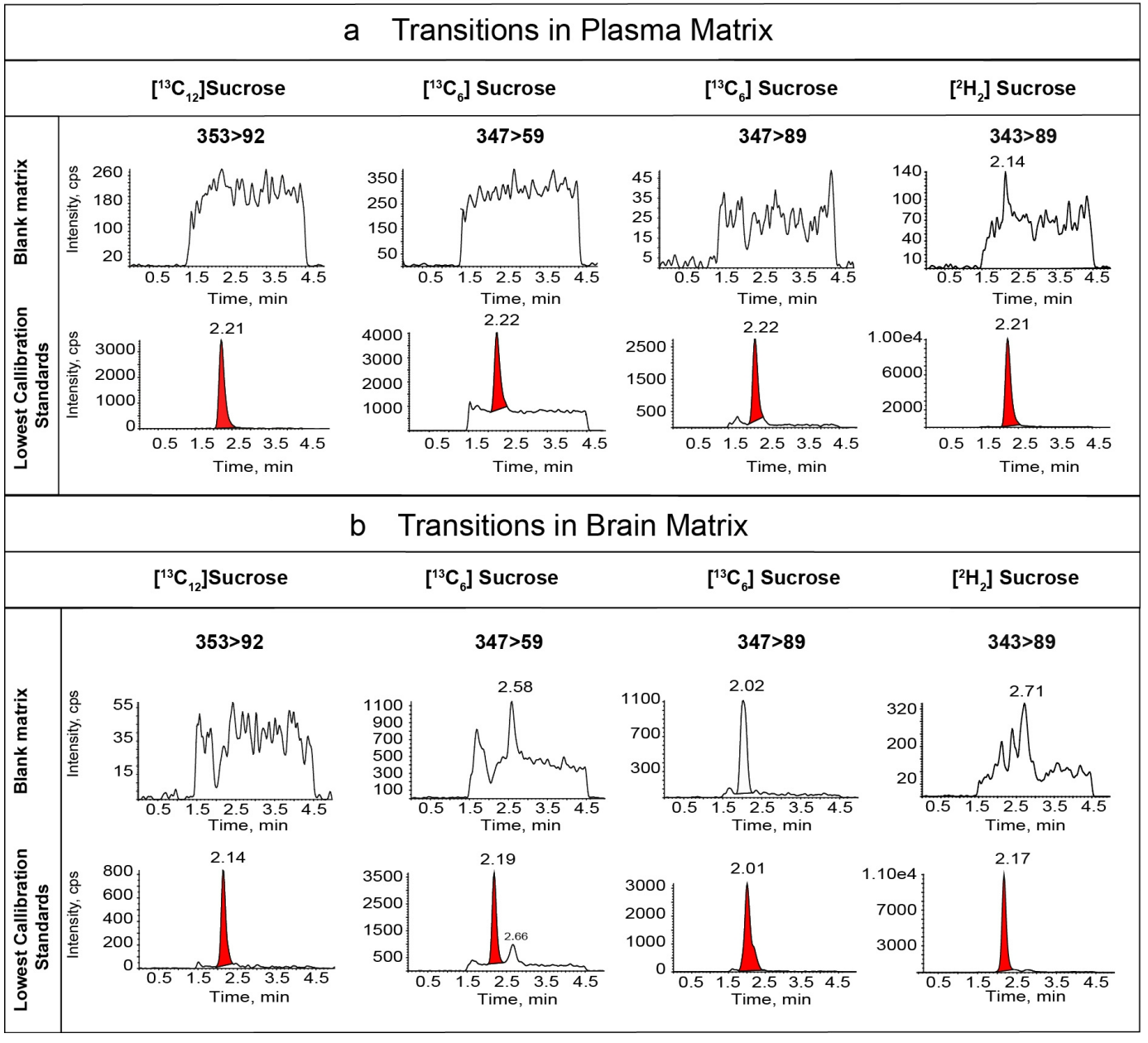
Figure 5: Freeze thaw stability of [$^{13}C_{12}$] sucrose and [$^{13}C_6$] sucrose in LC-MS/MS grade water. Columns and error bars represent mean and SD values, respectively (n=3).

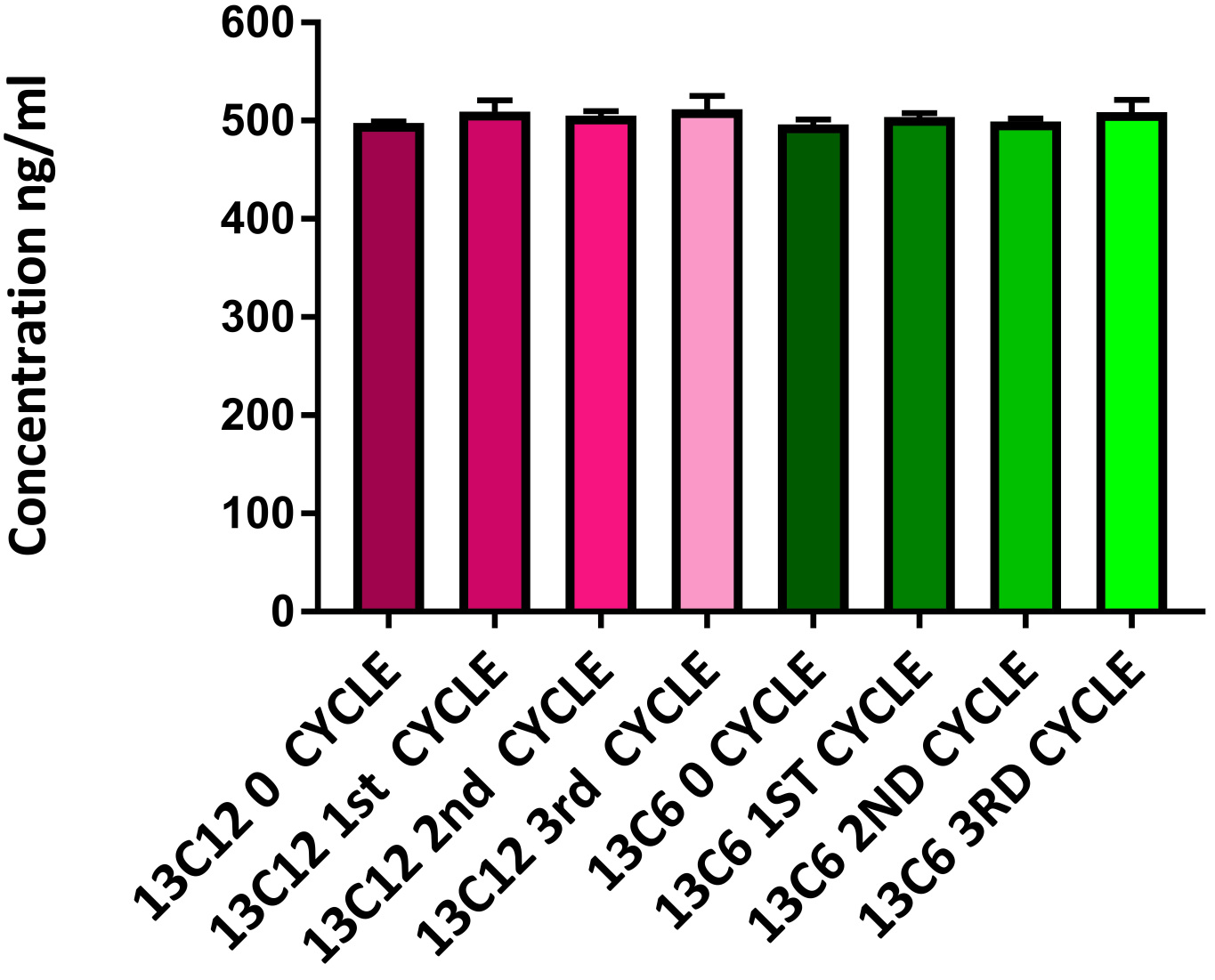
Figure 6: (a) Pharmacokinetic profile for [$^{13}C_{12}$] Sucrose up to 15 minutes in mouse plasma (mean ± SD, n=6). (b) Differences in brain concentrations of [$^{13}C_{12}$] Sucrose with or without correction by vascular marker (c) Differences in Kin values of [$^{13}C_{12}$] Sucrose with or without correction by vascular marker.

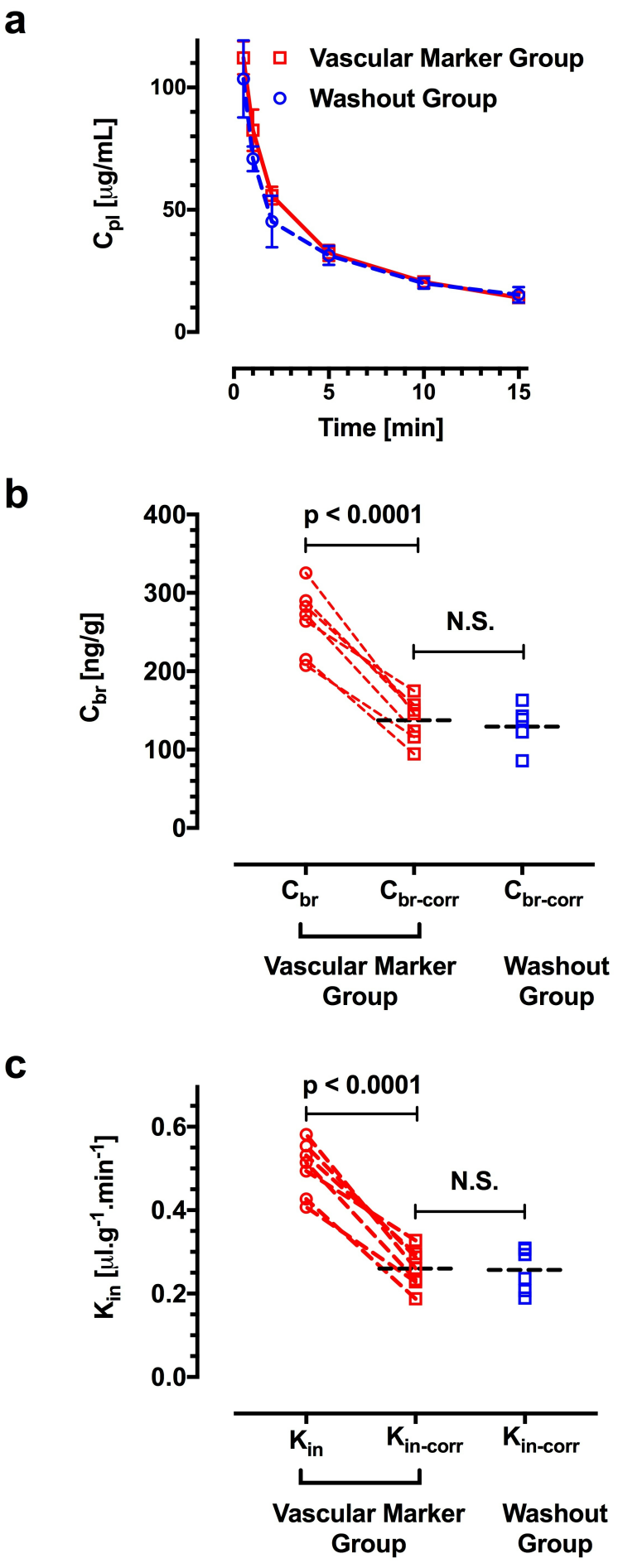

a
[13C12] Sucrose Mass Spectra
92.0
Product ion
Precursor ion
353.3
185.0
122.9
73.9
166.9
136.1
Intensity, cps
m/z, Da
b
[13C6] Sucrose Mass Spectra
89.3
Product ions
58.8
71.1
119.2
101.0
282.2
Precursor ion
347.2
m/z, Da
c
[2H2] Sucrose Mass Spectra
88.9
Product ion
Precursor ion
343.1
118.8
179.0
101.2
281.0
297.0
m/z, Da


Peak Area
3×10^5
2×10^5
1×10^5
0
[13C6]sucrose
[2H2]sucrose


Transitions in neat samples
353>92
347>59
343>89
347>89
Analyte
[13C12]Sucrose
[13C6] Sucrose
[2H2] Sucrose
Intensity, cps
Time, min
2.14
2.11
7500
3500
300
200
100
140
100
60
20
55
35
15
5000
3000
1000
4000
2000
500
100
45
25
5
1.10e4
7000
3000
0.5
1.5
2.5
3.5
4.5


a Transitions in Plasma Matrix
[13C12]Sucrose
[13C6] Sucrose
[13C6] Sucrose
[2H2] Sucrose
353>92
347>59
347>89
343>89
Blank matrix
Lowest Callibration Standards
Intensity, cps
Time, min
2.14
2.21
2.22
2.22
2.21
b Transitions in Brain Matrix
[13C12]Sucrose
[13C6] Sucrose
[13C6] Sucrose
[2H2] Sucrose
353>92
347>59
347>89
343>89
Blank matrix
Lowest Callibration Standards
Intensity, cps
Time, min
2.58
2.02
2.71
2.14
2.19
2.66
2.01
2.17


Concentration ng/ml
600
500
400
300
200
100
0
13C12 0 CYCLE
13C12 1st CYCLE
13C12 2nd CYCLE
13C12 3rd CYCLE
13C6 0 CYCLE
13C6 1ST CYCLE
13C6 2ND CYCLE
13C6 3RD CYCLE


a
Vascular Marker Group
Washout Group
C_{pl} [µg/mL]
100
50
0
0
5
10
15
Time [min]
b
p < 0.0001
N.S.
C_{br} [ng/g]
400
300
200
100
0
C_{br}
$C_{br-corr}$
$C_{br-corr}$
Vascular Marker Group
Washout Group
c
p < 0.0001
N.S.
K_{in} [µl.g^{-1}.min^{-1}]
0.6
0.4
0.2
0.0
K_{in}
$K_{in-corr}$
$K_{in-corr}$
Vascular Marker Group
Washout Group