Flow Injection with Chemical Reaction Interface–Isotope Ratio Mass Spectrometry: An Alternative to Off-Line Combustion for Detecting Low Levels of Enriched ^{13}C in Mass Balance Studies

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We have evaluated the potential of flow injection chemical reaction interface isotope-ratio mass spectrometry to replace radioactive labeling techniques in material balance studies. A sample is flow injected and transmitted through a desolvation system followed by combustion to form $^{13}CO_2$ with a microwave-powered chemical reaction interface. We can detect trace amounts of a ^{13}C-labeled drug (3′-azido-3′-deoxythymidine, AZT) in urine or feces. Our ability to quantify less than 100 ng/mL of excess ^{13}C (~1 μg/mL of ^{13}C-labeled AZT) from a sample equivalent to 10 μL of urine is superior to previous detection limits for ^{13}C in urine that use off-line combustion methods. Parallel studies using ^{14}C-labeled AZT showed that our stable isotope method provides comparable percent excretion data for urine and feces. These results support previous findings that mass balance studies could be carried out with isotope-ratio mass spectrometer, here using doses as low as 1–2 mg/kg. (J Am Soc Mass Spectrom 1999, 10, 153–158) © 1999 American Society for Mass Spectrometry

Despite the increasing emphasis on the use of stable isotopes, one necessary component of preclinical and clinical drug testing that continues to rely on radioisotope methodology is the material balance study. In such a study, one needs to know the percent of a drug that is found in a given biological fluid or tissue, independent of whether the drug exists in its original form or as any metabolite. This need has dictated the use of radioisotopes that have both low limits of detection and compound-independent sensitivity factors. Over the years, some attempts have been made to use stable isotope methodology and an isotope-ratio mass spectrometer (IRMS), but these preliminary studies do not appear to have been followed up by the pharmaceutical industry.

The first study of this type was conducted by von Unruh et al. [1]. They used the conventional method where a sample of urine was combusted and the resulting CO_2 was admitted into an IRMS. Their studies of variability in the baseline for the $^{13}C/^{12}C$ ratio from urine showed only small [SD $\delta^{13}C_{PDB} \simeq 1‰$ (this terminology is explained in the Methods section)] fluctuations over a 10 day period. This was reassuring because variations in diet will be reflected as variations in the baseline and could significantly limit the utility of stable isotope methods where differences below 1‰ can be measured. Knowing that the reproducibility of ^{13}C ratios of urine was good enough to permit this type of experiment, one subject was given a 0.32 mg dose of ^{13}C-labeled aspirin along with a 320 mg dose of unlabeled aspirin. The urine results showed a $\delta^{13}C$ value 2.8‰ above the control $^{13}C/^{12}C$ ratio using a 1% aliquot (8–16 mL) of a 24 h human urine sample. This elevated ratio converts to detecting an 83 ± 52% excretion of the administered ^{13}C aspirin dose into urine.

Nakagawa et al. [2] measured $\delta^{13}C$ and $\delta^{15}N$ in rat urine, feces, liver, and hair, again by combustion and measurement of the resulting CO_2 and N_2 by IRMS. Like von Unruh et al. [1], they found low baseline variations in both $\delta^{13}C$ and $\delta^{15}N$ values for blood, urine, feces, liver, and hair (SE ≤ 0.5%) and reported a material balance study with about ±10‰ reproducibility following a 40 mg/kg dose of ^{13}C, $^{15}N_2$-antipyrine.

Browne and co-workers [3] also examined this question, but using a different experimental approach. Here, a gas chromatograph was used to time focus the CO_2 generated from off-line combustion, and to separate CO_2 from N_2 and H_2O. For untreated urine they could detect ~4 μg/mL of a $^{13}C_2$-labeled drug. When high performance liquid chromatography (HPLC) was used to capture the one peak of interest that contained their

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labeled drug, their sensitivity improved to 0.015 μg/mL.

We approached this problem by using liquid flow injection to examine the potential of stable isotopes in material balance studies. The system used the chemical reaction interface mass spectrometry (CRIMS) technique that we recently adapted to an IRMS [4]. Following flow injection, CO_2 will be produced and transmitted to the IRMS in a steady stream of helium for the duration of the injection period. Flow injection provides efficient sample utilization and flexibility in the size of the sample analyzed. Our preliminary work with aminopyrine [5] spiked into human urine generated a nonspecific extraction scheme that contained all the organic carbon in urine and provided detection limits below 0.05 μg/mL of excess ^{13}C. The present study examines this approach in an *in vivo* experiment with 3′-azido-3′-deoxythymidine (AZT) in rats.

Materials and Methods

Chemicals

Methanol and water with less than 0.1 ppm evaporation residue were obtained from EM Science (Gibbstown, NJ). AZT, methyl-^{13}C (99% labeled) was obtained from Cambridge Isotope Laboratories (Andover, MA) and AZT-2-^{14}C (≥98%) was obtained from Moravek Biochemicals, Inc. (Brea, CA). 2′-Deoxycytidine 5′-monophosphate (dCMP ≥98%) was obtained from Sigma Chemical Co. (St. Louis, MI). Isopropanol and potassium carbonate A.C.S. grade were obtained from Fisher Scientific (Pittsburgh, PA).

Instrumentation

The general configuration of the instrument used in these experiments has been described before [4]. It consists of a HPLC pumping system coupled to an IRMS with a unique interface. The mass spectrometer used was a Finnigan/MAT delta S IRMS (San Jose, CA) with differential turbopumping and a triple Faraday cup collector. A Vestec Universal Interface (Vestec Mass Spectrometry Products, PerSeptive Biosystems, Framingham, MA) was used to couple the liquid flow from the HPLC pumps to the CRI [6] before passing into the IRMS. A split between the CRI and the IRMS that reduced the incoming gas flow as much as 1:10 was used. The CRI reactant gas in these experiments was oxygen [7].

Flow Injection

All experiments were conducted using a pair of Isco Model 260D syringe pumps (Isco Inc., Lincoln, NE) coupled with a Gilson Model 811C dynamic mixer. The mobile phase was 1/1 methanol/water (v/v) at a flow rate 1 mL/min. All samples were dissolved in 1/1 methanol/water and injected using a Rheodyne 7125 valve. Loop sizes of 20–5000 μL were tested and we found no significant differences in precision among them except for the 20 μL loop that led to a higher SD. With a 100 μL loop, the injected volume was between 25 and 40 μL that provided an m/z 44 signal of about 5 V.

Calculations and Standardization

The $^{13}C/^{12}C$ ratio is defined as the relative difference between the sample and a standard, calculated as

$$\delta^{13}C(‰) = [(R_{SPL} - R_{STD})/R_{STD}] \cdot 1000$$

where R_{SPL} refers to the ^{17}O-corrected $^{13}CO_2/^{12}CO_2$ ratio of the sample and R_{STD} refers to the same corrected ratio for the standard, Pee Dee Belemnite (PDB). (Pee Dee Belemnite no longer exists. Secondary standards with previous calibrations against PDB are currently used.) Oxidative CRI chemistry generates NO_2 that adds to the m/z 46 signal. Thus, m/z 46 cannot be used to measure the $^{12}C^{16}O^{18}O$ content of the sample, a quantity from which the ^{17}O content is computed and a correction made to obtain the true $^{13}C^{16}O_2$ signal at m/z 45. Without this correction, there will be an unknown error in the accuracy of the $\delta^{13}C_{PDB}$ values we compute. With CRIMS, the excess O_2 used as the reactant gas, rather than the trace amounts of oxygen in any analyte, dictates the isotopic composition of the oxygen in CO_2. While we cannot compute an accurate $\delta^{13}C$ value from the observed m/z 44 and 45 channels, the error will be constant because the isotopic composition of the oxygen is constant. Changes in the 45/44 ratios between samples will correctly represent changes in the ^{13}C content. To be formal, our calculations use the notation PDB*, where the asterisk denotes the absence of this correction [4]. In each calculation, the isotope ratio of our standard (dCMP that we determined had a $^{13}C_{PDB*}$ of −25.19‰ ± 1.13 via a chain of measurements ultimately linked to PDB) was used as R_{STD}, and the isotope ratio of each unknown peak was calculated from that known value. The integration of the m/z 44 and 45 channels following each injection of either a sample or the isotope ratio standard dCMP used the Isodat software with a slope sensitivity of 1 mV/s, which we found gave the most reproducible values.

Animal Experiments

Three male Sprague–Dawley rats weighing ~300 g were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA). Animals were housed one per metabolic cage at room temperature (~25 °C) on a 14 h light cycle. One batch of Purina lab chow was used throughout a 3 week control period and during the experiments. The chow and water were provided *ad libitum*.

One milligram of ^{13}C-AZT and 1 μCi of ^{14}C-AZT were dissolved in H_2O and injected into each animal intraperitoneally. The animals were held without food

for 24 h while collecting their urine and feces. After this period the urine volumes were measured and 5 mL were saturated with K_2CO_3 and extracted with an equal amount of isopropanol [8]. The fecal samples were homogenized in a blender with 15 mL of H_2O, saturated with K_2CO_3 and extracted with 15 mL of isopropanol. The isopropanol extracts were used to quantify AZT and its metabolites. The isopropanol extracts were diluted ten-fold with methanol/H_2O (1/1) and filtered with a 0.45 μm Cameo Teflon filter (Micron Separations, Westborough, MA).

Mass Balance using Radioactivity

One microcurie of ^{14}C-AZT was dissolved in 15 mL of H_2O. Spikes of 0, 3, 6, 9, and 12 μL of the stock solution were added into 7 mL scintillation vials (Fisher brand, Fisher Scientific, Pittsburgh, PA) containing 4 mL of Scintiverse BD scintillation counting cocktail (Fisher Scientific). Ten microliters of the urine or fecal extracts for rats 1, 2, and 3 were added to scintillation vials with 4 mL of Scintiverse BD. In each case the vials were vortexed for 10 s. Standard solutions and extracts were counted in a Beckman LS 6500 Multipurpose Scintillation Counter (Beckman Instruments, Inc., Columbia, MD). The counting time for each sample was 1 min and the ^{14}C disintegrations per minute (D.P.M.) for each sample was recorded.

Mass Balance using Stable Isotopes

Urine extracts from rats 1, 2, and 3 and a fecal extract from rat 1 were analyzed with 5–6 replicate injections. A standard addition graph was generated for each sample. To accomplish this, we spiked 100 μL of extracted urine and feces with 100 μL of aqueous solutions containing various amounts of ^{13}C-AZT and diluted to 1 mL with mobile phase. Based on the ^{14}C-AZT studies, we knew approximately how much ^{13}C-AZT to expect in each sample. For urine and fecal extracts from rat 1, 0.50, 1.0, 2.0, 3.0, 4.0, and 5.0 μg of ^{13}C-AZT were added. For urine extracts from rats 2 and 3, 1.0, 3.0, 5.0, 10.0, 15.0, and 20.0 μg of ^{13}C-AZT were added. The spiked extracts were diluted to 1 mL and analyzed by flow injection/CRI/IRMS. Their respective δ values were recorded and the ^{13}C-AZT concentration of the urine and feces were calculated. Between 10 and 50 μL of dCMP (1 mg/mL) were flow injected before each isopropanol extract to calibrate the isotope ratio scale. The results are expressed as $\Delta\delta^{13}C$, because the differences in isotope ratios between unspiked and spiked urine samples are plotted rather than their absolute values.

Results and Discussion

To analyze a sample in a mass balance study, it is imperative that the method is nonselective so that all labeled species are determined, whatever their chemical nature. For this reason, radiometric methods have been standard because they fulfill this criterion. Using HPLC/CRI/IRMS, the detection of ^{13}C is general, but, unlike radiometric detection, the matrix can present problems because of nonvolatile species that are present at high concentrations. Consequently, the preliminary phase of our research evaluated how samples were to be processed. The saturated K_2CO_3/isopropanol method was quantitative both for organic carbon from urine and for the aminopyrine that was our test species. While the pH of a saturated K_2CO_3 solution is 11.6, this extraction process is not limited to basic and neutral species. In the original work by Horning et al. [8], both phenobarbital (a weak acid) and caffeine (neutral) were extracted with 100% efficiency from plasma as measured with ^{14}C-labeled drugs. That procedure used only 1 mL of isopropanol to extract from 6 mL of diluted plasma. Here, we used equal amounts of isopropanol and aqueous sample that should have improved the extraction for less readily extracted species.

In setting up the Universal Interface (UI), one works at relatively low (50 °C) temperatures in the countercurrent gas diffusion cell to retain marginally volatile analytes or can elevate the temperature to achieve better desolvation and prevent condensation. After the initial years of using the UI, we felt that temperatures greater than 50 °C markedly improved long-term reliability and became the standard for our operation. A downside was that aminopyrine was sufficiently volatile that it was lost. Volatile drugs and volatile metabolites represent problems for many HPLC/MS interfaces because the process of desolvating the liquid phase may also evaporate analytes. Caffeine is another rather volatile analyte for which we have noted inconsistent behavior. Clearly, a HPLC-style interface may be inappropriate for a mass balance study if there is a chance of volatile metabolites.

Because of this problem, we switched our experiments to AZT. AZT is one of the few drugs that is commercially available with both radio- and stable-isotope carbon labeling. Even so, the ^{13}C label is on a different position than the ^{14}C label, but the metabolism of AZT does not appear to involve either of the positions labeled [9]. A full validation of stable-isotope methodology for conducting a material balance study would involve administering both a radio-labeled and stable-isotope-labeled test molecule and comparing the elimination of the drug given. With that in mind, the animals received an injection containing both ^{14}C AZT and ^{13}C AZT after which the total urinary and fecal outputs were collected for 24 h and then extracted. The samples were first examined by scintillation spectrometry so that the fractional excretions could be immediately determined. Two of the three fecal samples were below what we anticipated our limit of quantitation with CRIMS to be: rat 2 was 1.4% and rat 3 was 1.2% of the administered dose.

To determine the excess ^{13}C for the remaining samples we used the method of standard additions [10].

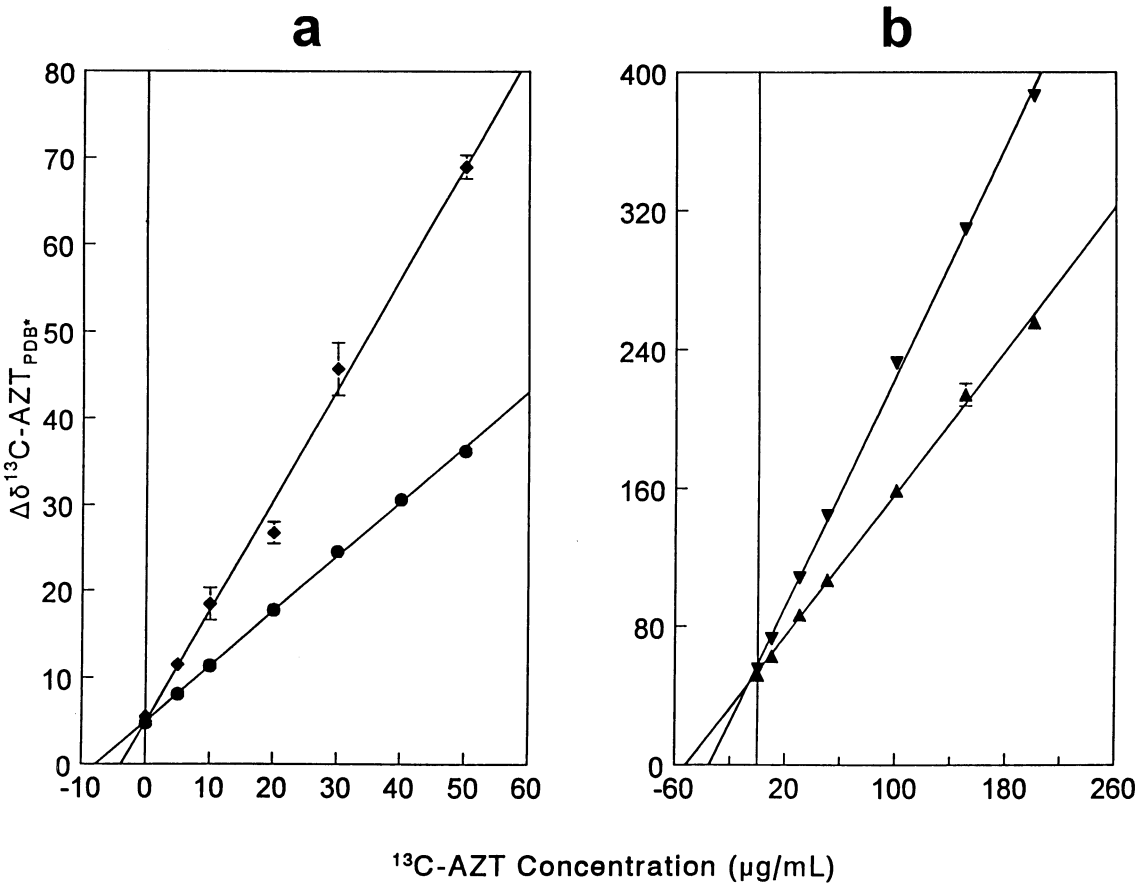

Figure 1. Standard additions: The $\Delta\delta^{13}$C-AZT$_{PDB^*}$ is plotted against various concentrations (μg/mL) of ^{13}C-AZT spiked in extracted urine and feces. **(a)** rat 1: closed circle = fecal extract; closed diamond = urine extract. **(b)** rat 2 = closed triangle and rat 3 = closed inverted triangle; urine extracts. The errors are standard deviations resulting from six replicate injections. The unweighted linear regression lines are also shown extrapolated to $y = 0$.

Equal volumes (100 μL) of either extracted urine or feces were spiked with increasing amounts of ^{13}C AZT and then diluted to 1 mL. Data were plotted and the linear regression line was drawn. These standard addition graphs are seen in Figure 1a, b. For rat 1 urine, the standard addition graph gave $r^2 = 0.993$ with a $y = 0$ extrapolation that indicated 58 $\pm$ 18 (SE) μg of ^{13}C AZT was excreted or 5.8 $\pm$ 1.8% of the administered dose. The calculation was done by multiplying the x intercept by the total volume of urine, which was 15 mL for rat 1 and rat 2, and 23 mL for rat 3. The graph was inverted ($x' = \Delta\delta^{13}C_{PDB^*}$ and $y' = \mu$g/mL ^{13}C AZT) and another regression was done. The purpose of this second regression was to obtain the relative standard error of the μg/mL ^{13}C AZT parameter.

The regression of standard additions to rat 2 urine gave an $r^2 = 0.998$ and the x intercept indicated that 783 $\pm$ 41 (SE) μg of ^{13}C AZT (or 78 $\pm$ 4.1% of the administered dose of ^{13}C AZT) was present. Analysis of rat 3 urine gave an $r^2 = 0.999$ AZT and a computed excretion of 807 $\pm$ 46 (SE) μg ^{13}C AZT (or 81 $\pm$ 4.6% ^{13}C AZT). The standard additions to the extract of feces from rat 1 gave $r^2 = 0.999$ and 118 $\pm$ 6 (SE) μg of ^{13}C AZT excreted (11.8 $\pm$ 0.6% of the dose). The percentage recoveries of animal urine or feces labeled with either ^{14}C-AZT and ^{13}C-AZT are presented and compared in Table 1. Without conducting these experiments in many more animals, we cannot figure out whether the -13% to $+2.2\%$ differences between ^{14}C and ^{13}C are systematic, analytical, or random. These limited data do not prove that the CRI/IRMS method is capable of substituting for radiometric methods, but do show the ability of our approach to generate data from realistic samples that track the radiometric data. Experiments to fully validate a stable isotope method would have to use a drug with identical positions for the labels and would

Table 1. Comparing the percentage recovery AZT as measured by ^{14}C and ^{13}C

Animal	Sample	^{13}C (%) of dose	^{14}C (%) of dose
1	Feces	11.8	11.1
1	Urine	5.8	8.0
2	Urine	78	65
3	Urine	80	68

have to be repeated a sufficient number of times to provide statistical assurance of comparability.

The 0–24 h urinary excretion data for rats 2 and 3 are consistent with published results for AZT in mice, 86% [9] and rats, 78% [11]. Obviously, the data for rat 1 are not. We can only assume that the intraperitoneal dose for rat 1 entered the gut and not the peritoneal space. As a result, we observed much less drug in urine and significant amounts in feces, indicative of incomplete absorption. Ahmed et al. [9] found 4.6% AZT in feces after i.v. dosing while de Miranda et al. [11] found 20% in feces after administering the AZT by gavage showing that higher fecal excretion will occur through partial absorption. Our material balance for rat 1 is incomplete, but the imperfect nature of the experiment only allows speculation about why. Still, this "accident" allowed us to compare ^{13}C with ^{14}C for lower urinary excretion and a measurable amount of fecal excretion (12% ^{13}C AZT) than would have resulted from ideal dosing.

The $\delta^{13}C$ values from control urines were -29.42 ± 0.63, -30.75 ± 0.84, -30.34 ± 0.73 for rats 1–3, respectively (mean $\pm$ SD, N = 5) and the fecal control value was -30.18 ± 0.58. From these values, we computed the limits of quantitation (LOQ) based on the +2SD values times the total volume of urine divided by the slope of the regression line. This result was divided by the total amount of AZT given (1 mg) and then expressed as a percent. The LOQ from urine for rat 1 was 1.5% of the AZT dose administered; for rat 2, the LOQ was 2.4% of the AZT administered; and for rat 3 the LOQ was 2.0% of the dose. For feces, the LOQ in rat 1 was 2.7%. While we did not measure samples of rat urine containing ^{13}C-labeled AZT enriched at quite these low levels, it should be recognized that these estimated LOQs are not extrapolations but effective interpolations between the readily measured isotope ratio of control urine and a similar measurement from an enriched sample.

Our measurements provided better limits of detection than other work where the conversion of the sample to CO_2 was done with off-line combustion methods [1, 2]. The only case where a lower percentage excretion was quantified was in that part of the work by Browne et al. [3], where they extracted urine with a selective process and collected a particular HPLC peak containing the drug molecule of interest. In this way, they eliminated most of the background from the urine and could more selectively detect ^{13}C from the targeted analyte. When actually carrying out a recovery study for a drug where an unknown extent of metabolism and range of metabolites existed, the selection of specific components for IRMS analysis would not be acceptable.

Flow injection analysis was more efficient in both use of sample and better time management than the other methods used for material balance studies. In that part of the report by Brown et al. where they did not use a selective extraction and HPLC purification process [3], they introduced the equivalent of 10–20 μL of urine into their IRMS. We were able to analyze the equivalent of 12.5 μL of urine from our nonselective urine extract while obtaining better detection limits. The conventional approaches used by von Unruh et al. [1] and Nakagawa et al. [2] were yet less sensitive. The disadvantage of flow injection analysis was the requirement of nonselective extraction to avoid contaminating the apparatus with nonvolatile materials. In off-line combustion methods, the nonvolatile materials remain in the combustion apparatus and only the resulting gases are transferred to the IRMS.

Conclusions

Stable isotopes (e.g., ^{13}C) are rapidly replacing radioisotopes for drug metabolism studies. Using stable isotopes has the capacity to reduce: (1) specialized hazards of working with radioactivity, (2) the highly regulated and limited use of radioisotopes in humans, (3) the special problems in manufacturing radiolabeled compounds, and (4) waste disposal of radiolabeled biomedical waste. Our work extends the prospects for conducting a mass balance study. An extrapolation of our rat experiments to human studies is illustrative. Our typical LOQ was about 2% or 20 μg of ^{13}C-AZT in about 17 mL of urine or 1 μg/mL. Twenty micrograms of ^{13}C-AZT is the same as 1.0 μg of ^{13}C, so our limit for excess ^{13}C is somewhat less than 0.1 μg/mL. We assume a 70 kg person excretes 1200 mL of urine per day, so we could detect 120 μg of excess ^{13}C. While the calculation depends on the number of labels and the size of the drug molecule in which the label is incorporated, we would predict that 1% of a drug like $^{13}C_1$-AZT (MW = 268) given at a dose of 1–2 mg/kg would be quantifiable in urine. Extrapolating this level of detection to the general question of material balance studies suggests that many drugs of low and intermediate potency could be analyzed this way. Highly potent drugs would be given at lower doses than this and our approach to using stable isotopic labels would not be successful.

We recently showed that HPLC/CRIMS detected ^{13}C and ^{15}N labels in a drug metabolism study better than a radioactivity monitor detected ^{14}C [12]. A second favorable comparison of ^{14}C and stable isotopes was reported by Goldthwaite et al. [13]. The present study shows a different way that new methods of detecting stable isotopes make it feasible for them to be used in the pharmaceutical industry for experiments that have previously demanded the use of radioisotopic labels.

The broad question of how to optimally carry out mass balance studies with stable isotopes is not answered with this study. While our detection limits are superior to off-line combustion methods, the possibility of loss of volatile metabolites in the desolvation process puts continuous flow methods at a disadvantage. Even for radioisotopes, one must have concern for volatile metabolites, such as $^{14}CO_2$, that would be lost from a material balance study without special breath trapping. Further experiments comparing radioisotopes and stable isotopes are needed to better focus this question.

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