Development of a non-radiometric method for measuring the arterial input function of a $^{11}$C-labeled PET radiotracer

H. Umesha Shetty$^{1,3}$, Sami S. Zoghbi$^1$, Cheryl L. Morse$^1$, Aneta Kowalski$^1$, Jussi Hirvonen$^2$, Robert B. Innis$^1$ & Victor W. Pike$^1$

Positron emission tomography (PET) uses radiotracers to quantify important biochemical parameters in human subjects. A radiotracer arterial input function (AIF) is often essential for converting brain PET data into robust output measures. For radiotracers labeled with carbon-11 ($t_{1/2} = 20.4$ min), AIF is routinely determined with radio-HPLC of blood sampled frequently during the PET experiment. There has been no alternative to this logistically demanding method, neither for regular use nor validation. A $^{11}$C-labeled tracer is always accompanied by a large excess of non-radioactive tracer known as carrier. In principle, AIF might be obtained by measuring the molar activity ($A_m$; ratio of radioactivity to total mass; Bq/mol) of a radiotracer dose and the time-course of carrier concentration in plasma after radiotracer injection. Here, we implement this principle in a new method for determining AIF, as shown by using $[^{11}$C]PBR28 as a representative tracer. The method uses liquid chromatography-tandem mass spectrometry for measuring radiotracer $A_m$ and then the carrier in plasma sampled regularly over the course of a PET experiment. $A_m$ and AIF were determined radiometrically for comparison. The new non-radiometric method is not constrained by the short half-life of carbon-11 and is an attractive alternative to conventional AIF measurement.

Positron emission tomography (PET) is a uniquely valuable molecular imaging modality for noninvasively exploring physiology and biochemistry in health and disease$^{1,2}$, and has an expanding role in drug development$^3$ and medical diagnosis$^4$. PET has notable importance for neuropsychiatric research both for the study of pathophysiology and for drug development. Appropriately designed radiotracers$^5$ permit sensitive imaging and quantification of many of the proteins in brain$^6$ that are implicated in neuropsychiatric$^7$, neurological$^8$, and neurodegenerative disorders$^9$, as well as substance dependence$^{10}$. These proteins include various neurotransmitter receptors, transporters, enzymes, and amyloid plaques$^3$. PET radiotracers may also be used to verify protein target engagement by experimental drugs and how target engagement varies with dosing regimen$^{11}$. Such information can be critical for establishing meaningful clinical trials. Short-lived carbon-11 ($t_{1/2} = 20.4$ min) or fluorine-18 ($t_{1/2} = 110$ min) are the two most commonly used radionuclides for labeling radiotracers for PET imaging of brain$^3$.

Typically, in each PET scanning session, measurement of the radiometabolite-corrected arterial input function (AIF) of the radiotracer is required for use in conjunction with a biomathematical model to robustly quantify a radiotracer target within brain$^{12}$. Nearly all PET radiotracers generate radiometabolites in plasma$^{3}$. The AIF is the time-course of non-metabolized radiotracer in plasma. Virtually all AIF measurement has been based on a single methodology, namely fast radio-high performance liquid chromatography (radio-HPLC) separation of the parent radiotracer from radiometabolites in plasma from multiple blood samples taken serially throughout a PET scanning session for radiometric quantification. When imaging with a $^{11}$C-labeled PET tracer, the time available for measuring AIF by this means is severely constrained to a few half-lives (typically ~90 min). This is very logistically demanding. Whereas the dose of a radiotracer administered to a human subject (~ 750 MBq) is measured with an ionization chamber, the low levels of radioactivity found in plasma samples (of the order of kBq) are typically measured with a sensitive γ-counter. The accuracy of radioactivity measured with an ionization chamber or γ-counter depends, among other factors, on the surrogate radioisotope(s) used to calibrate

$^1$Molecular Imaging Branch, National Institute of Mental Health, National Institutes of Health (NIMH/NIH), Building 10, Room B3C351, 10 Center Drive, MSC 1003, Bethesda, MD 20892, USA. $^2$Department of Radiology and Turku PET Centre, University of Turku and Turku Central Hospital, 20520 Turku, Finland. $^3$email: shettyu@mail.nih.gov
Notably, no alternative method has ever been available to validate AIFs measured with the conventional radiometric technique. A method for AIF measurement that avoids the severe time constraint imposed by fast decaying radioactivity would be invaluable.

A ¹¹C-labeled PET radiotracer is always accompanied by a matching non-radioactive tracer known as carrier. Although the carrier mass is typically small (< 5 nmol) in any administered radiotracer dose, it is always much larger than the mass of the radiotracer, usually by about three orders of magnitude. In principle, AIF may be obtained by measuring the molar activity (\(A_m\); ratio of radioactivity to combined mass of tracer and carrier; Bq/mol) of a radiotracer dose and the time-course of the very low concentrations of carrier in plasma after radiotracer injection.

An earlier study from our laboratory demonstrated that liquid chromatography-tandem mass spectrometry (LC–MS/MS) has sufficient sensitivity to detect not only the non-radioactive isotopologues (i.e., ¹²C and ¹³C species) in a ¹¹C-labeled tracer but also the relatively low mass radioactive isotopologue (i.e., the ¹¹C species). Here, in this study, we further explored the use of sensitive LC–MS/MS to measure each of the three carbon isotopologues in a PET radiotracer dose, which we here denote [¹¹C]i, [¹²C]i, and [¹³C]i, and the application of such measurements to human plasma samples for AIF determination. We furthermore compared the resultant \(A_m\) and AIF data with those determined by the conventional radiometric method for the same ¹¹C-labeled radiotracer, using [¹¹C]PR28 as the principal example.

To develop an LC–MS/MS method for AIF determination, we needed to show that LC–MS/MS has (1) sufficient sensitivity to accurately measure the ratio of all three isotopologues in a radiotracer dose, (2) adequate sensitivity and specificity to measure the low level of carrier from the radiotracer in human plasma after intravenous administration of the radiotracer at a useful molar activity, especially over the whole time course of PET imaging (typically 90 min), and (3) that the obtained AIF is reliable and accurate. We performed detailed experiments to establish these aims.

**Results**

**LC–MS/MS investigation of the carrier in ¹¹C-labeled tracers: ¹³C to ¹²C ratio at the radiolabelling site and dependence of this ratio on \(A_m\)**. As part of method development, we set out to measure the ratio of ¹³C to ¹²C in the carrier of some ¹¹C-labeled tracers to determine whether there was any variability that might impact on the proposed use of LC–MS/MS for measuring \(A_m\) and AIF. We showed that we could measure simultaneously all three isotopologues, [¹¹C]i, [¹²C]i, and [¹³C]i, in radiotracers with high \(A_m\) values and, two isotopologues, [¹¹C]i and [¹³C]i, when \(A_m\) is in low to moderate range. The ratio of ¹³C to ¹²C in the natural environment is about 1.1% but shows small variations that depend on carbon source. For the determination of \(A_m\) values, we proposed to measure two isotopologues only, [¹¹C]i, and [¹³C]i. This approach avoids detector saturation with the much more abundant [¹²C]i, especially for samples with \(A_m\) at the lower end of the normal range. To calculate the amount of [¹³C]i from a measurement of [¹¹C]i, their exact ratio in a sample of the compound would need to be known. This ratio was first measured in natural abundance reference compounds for four of the studied PET radiotracers (specifically, three TSPO radiotracers—[¹¹C]PBR28, [¹¹C](R)-PK11195, and [¹¹C]DPA713—and one cyclic adenosine monophosphate (cAMP) phosphodiesterase-4 (PDE-4) radiotracer, [¹¹C](R)-rolipram). The obtained values were within the expected range of about 1.1% per carbon atom in a measured product ion (reference standards, Table 1).

However, when this method was applied to the carrier present in prepared radiotracer doses, we observed [¹³C]i to [¹²C]i ratios that were well above the normal range for natural abundance (Table 1). These findings showed that ¹³C enrichment had occurred during radiotracer production, which we now attribute to a known nuclear reaction, \(^{14}\text{N}(p,2n)^{13}\text{C}\), that would co-exist with the \(^{14}\text{N}(p,α)^{11}\text{C}\) reaction during the cyclotron production of carbon-11 through irradiation of nitrogen with 16 MeV protons (see “Discussion” section). In our production setting, the \(A_m\) value of a PET radiotracer strongly reflects the amount of radioactivity produced during
Cyclotron irradiation, which in turn depends on the integrated proton beam current (μA × min)\(^2\). Consequently, we predicted that the \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio would increase with the measured \(A_m\) value of the \(^{11}\text{C}\)-labeled tracer.

In each of four radiotracers, when measuring the fragment ion that contained the radiolabeling site in two separately prepared doses, the \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio was higher in the dose that had the higher \(A_m\) value (determined radiometrically) (Table 1). One radiotracer \((R)\)-PK11195 gave abundant product ions (\(m/z\) 238; 239) that lacked the \(^{11}\text{C}\)-labeling site (i.e., lacked the amido \(N\)-methyl group). The \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio for these ions was found to be in the range of natural abundance and invariant with the \(A_m\) value of the radiotracer preparation (Table 1), thereby affirming that changes in \(^{13}\text{C}\)-enrichment were confined to the fragment containing the \(^{11}\text{C}\)-labeling site.

### Table 1.

| Radiotracer | Ions measured \((m/z)\) | Number of carbons in product ion | \(A_m\) (GBq/μmol)\(^a\) | Ratio of \[^{13}\text{C}\] to \[^{12}\text{C}\] (\%)\(^b\) |
|------------|------------------------|---------------------------------|---------------------------|-----------------|
| PBR28      | 121, 122 \(m/z\) 121;122 | 8                               | 367                       | 9.12 ± 0.08\(^c\) |
|            |                        |                                 | 876                       | 9.89 ± 0.02     |
|            |                        |                                 |                           | 10.98 ± 0.11    |
| \((R)\)-Rolipram | 191, 192 \(m/z\) 191;192 | 11                              | 178                       | 12.48 ± 0.07\(^c\) |
|            |                        |                                 | 1194                      | 13.12 ± 0.07    |
|            |                        |                                 |                           | 14.30 ± 0.11    |
| DPA713     | 266, 267 \(m/z\) 266;267 | 16                              | 433                       | 18.72 ± 0.04\(^c\) |
|            |                        |                                 | 566                       | 19.91 ± 0.04    |
|            |                        |                                 |                           | 20.17 ± 0.10    |
| \((R)\)-PK11195 | 297, 298 \(m/z\) 297;298 | 17                              | 483                       | 19.98 ± 0.18\(^c\) |
|            |                        |                                 | 618                       | 20.94 ± 0.21    |
|            |                        |                                 |                           | 21.03 ± 0.18    |
|            | 238, 239 \(m/z\) 238;239 | 15                              | 483                       | 17.10 ± 0.16\(^c\) |
|            |                        |                                 | 618                       | 17.09 ± 0.12    |
|            |                        |                                 |                           | 17.23 ± 0.08    |

\(^a\) Determined with radio-HPLC.  
\(^b\) Mean ± SD of four LC–MS/MS measurements; ratio includes minor contribution from the natural abundances of \(^2\text{H}\), \(^17\text{O}\), and \(^{15}\text{N}\).  
\(^c\) \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio for the reference non-radioactive compound.  
\(^d\) From fragmentation \(a\).  
\(^e\) From fragmentation \(b\).

Cyclotron irradiation, which in turn depends on the integrated proton beam current (μA × min)\(^2\). Consequently, we predicted that the \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio would increase with the measured \(A_m\) value of the \(^{11}\text{C}\)-labeled tracer. In each of four radiotracers, when measuring the fragment ion that contained the radiolabeling site in two separately prepared doses, the \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio was higher in the dose that had the higher \(A_m\) value (determined radiometrically) (Table 1). One radiotracer \((R)\)-PK11195 gave abundant product ions \((m/z\) 238; 239\) that lacked the \(^{11}\text{C}\)-labeling site (i.e., lacked the amido \(N\)-methyl group). The \(^{13}\text{C}\) to \(^{12}\text{C}\) ratio for these ions was found to be in the range of natural abundance and invariant with the \(A_m\) value of the radiotracer preparation (Table 1), thereby affirming that changes in \(^{13}\text{C}\)-enrichment were confined to the fragment containing the \(^{11}\text{C}\)-labeling site.
The data from Table 1 were used to plot the increase in the ratio of $^{[13]C}_i$ to $^{[12]C}_i$ for the carrier in all eight radiotracer productions versus $A_m$ value. These values and the corresponding $A_m$ values were found to be strongly correlated ($r = 0.895; p < 0.003$) (Supplementary Fig. S1).

**Determination of radiotracer $A_m$ by LC–MS/MS alone.** $A_m$ values for $^{[11]C}$PBR28 preparations were measured with LC–MS/MS by isolating and monitoring the isotopologue pair, $^{[11]C}_i$ and $^{[13]C}_i$, as previously described for $^{11}$C-labeled tracers. In that study, the ratio of $^{[13]C}_i$ to $^{[12]C}_i$ was taken to be the fixed value measured in reference PBR28. Here, in a refinement of this procedure, the ratio of $^{[13]C}_i$ to $^{[12]C}_i$ in the carrier of each $^{[11]C}$PBR28 preparation was used to calculate the $^{12}$C-peak area from which $A_m$ values could then be derived. In the case of a few $^{[11]C}$PBR28 preparations, where the $A_m$ values were relatively high (905–1124 GBq/µmol), LC–MS/MS successfully isolated and monitored all three carbon isotopologues simultaneously ($^{[11]C}_i$, $^{[12]C}_i$, and $^{[13]C}_i$) (Supplementary Fig. S2). This direct measurement of all three types of isotopologue yielded $A_m$ values that closely matched those obtained by measuring only the two isotopologues $^{[11]C}_i$ and $^{[13]C}_i$. Therefore, measurement of $^{[11]C}_i$ and $^{[13]C}_i$ alone plus a separate measurement of the $^{13}$C to $^{12}$C ratio in a radiotracer dose sufficed to provide an accurate $A_m$ value.

**Plot of $^{[13]C}$ to $^{[12]C}$ ratio versus $A_m$ (by LC–MS/MS) for $^{[11]C}$PBR28.** $A_m$ values, including the ratio of $^{[13]C}_i$ to $^{[12]C}_i$, were determined in 16 preparations of $^{[11]C}$PBR28. Variations in radiosynthesis time were negligible (36.3 ± 0.82 min; mean ± SD; $n = 16$) and dose radioactivity was therefore decay-corrected to the end of each synthesis. As a control, the ratio of $^{[13]C}_i$ to $^{[12]C}_i$ was also measured in reference (natural abundance) PBR28 on each occasion of radiotracer analysis. The ratios of $^{[13]C}_i$ to $^{[12]C}_i$ for $^{[11]C}$PBR28 preparations correlated strongly with $A_m$ values determined with LC–MS/MS ($r = 0.975; p < 0.0001; n = 16$) (Fig. 2). The $Y$-axis intercept of 9.09% ($A_m = 0$) for this curve was almost identical to the mean ratio of $^{[13]C}_i$ to $^{[12]C}_i$ measured for reference PBR28 (9.16 ± 0.05%; mean ± SD; $n = 16$; represented by the red line in Fig. 2). The small standard deviations in the latter value and those in Table 1 demonstrate the high precision with which such ratios could be determined with LC–MS/MS.

**Comparison of LC–MS/MS and radiometric methods for measuring $A_m$.** The $A_m$ values of each of 15 preparations of $^{[11]C}$PBR28 were measured in three different ways: with LC–MS/MS alone (Method 1); with carrier measured with LC–MS/MS and radioactivity measured in a calibrated $\gamma$-counter (Method 2), and with the conventional radiometric method based on radioactivity dose measured in an ionization chamber and carrier measured with HPLC (Method 3) (Supplementary Methods). A scatter plot of the percentage difference between the $A_m$ values measured with LC–MS/MS alone and each of the two radiometric methods is displayed in Supplementary Fig. S3. When compared with LC–MS/MS (Method 1), measurement with an ionization chamber (Method 2) gave 1.5 ± 10.1% lower $A_m$ values, and measurement with a $\gamma$-counter (Method 3) gave 33.2 ± 3.3% higher $A_m$ values.

**Comparison of radioactivity measurements made with LC–MS/MS with those of $\gamma$-counter.** Radioactivity in a sample of $^{[11]C}$PBR28 was measured with a calibrated $\gamma$-counter. The carrier $^{[12]C}_i$ in the same sample was then quantified with LC–MS/MS using an internal standard of $^{[15]C}_2H_3$PBR28 to calibrate the LC–MS/MS response. Radioactivity based on the mass of $^{[11]C}_i$ was calculated using the concentra-
A value of [11C]PBR28 determined with LC–MS/MS. Measurement with a γ-counter gave 31.8 ± 3.8% (n = 24) higher radioactivity values than LC–MS/MS. Data from the analysis of six [11C]PBR28 preparations are shown in Table 2. The substantial difference between the radioactivity obtained by direct counting and through the mass of [11C]i was independent of the A_m value (200–1028 GBq/µmol) and also independent of whether two isotopologues ([11C]i and [13C]i) or three isotopologues ([11C]i, [12C]i and [13C]i) were analyzed. Thus, differences in radioactivity estimates from γ-counting and LC–MS/MS accounted for all the A_m discrepancies shown in Supplementary Fig. S3b.

**Table 2.** Comparison of [11C]PBR28 radioactivity data from γ-counter and from LC–MS/MS. a Measured using [13C,2H3]PBR28 as an internal standard. b A_m value by LC–MS/MS of [11C], and [13C], (1–3), and of the triad [11C]i, [12C]i, and [13C]i, (4–6). c Radioactivity calculated from the A_m and carrier, each measured with LC–MS/MS. d From that determined with LC–MS/MS.

| [11C]PBR28 preparation | Carrier by LC–MS/MS (pmol) a | A_m by LC–MS/MS (GBq/µmol) b | Radioactivity by LC–MS/MS (kBq) c | γ-counter (kBq) | Difference in radioactivity (%) d |
|-------------------------|-------------------------------|-----------------------------|---------------------------------|----------------|---------------------------------|
| 1                       | 15.2                          | 200.4                       | 3040                            | 4019           | 32.2                            |
| 2                       | 5.56                          | 631.1                       | 3510                            | 4817           | 37.2                            |
| 3                       | 12.1                          | 383.5                       | 4645                            | 6310           | 35.8                            |
| 4                       | 1.75                          | 904.9                       | 1580                            | 2092           | 32.4                            |
| 5                       | 2.90                          | 1124                        | 3261                            | 4249           | 30.3                            |
| 6                       | 2.95                          | 1028                        | 3036                            | 4150           | 36.7                            |

**Figure 3.** LC–MS/MS ion chromatograms ([m/z = 121; [13C]) for carrier PBR28 in arterial plasma sampled from a human subject injected intravenously with [11C]PBR28 for PET imaging. a1: baseline. Peaks a2 and a3: carrier PBR28 peaks at 10 min (71.8 pM) and 90 min (6.34 pM) after injection, respectively. Peak b in ion chromatogram ([m/z = 125) is from the analogous transition in the internal standard, [11C,2H3]PBR28 (284 pM).

**LC–MS/MS of carrier in human plasma.** By using [13C,2H3]PBR28 as an internal standard, a LC–MS/MS method was developed to quantify the very low amounts of PBR28 carrier in the plasma of eight human subjects who had been injected intravenously with [11C]PBR28 for PET scanning. Figure 3 shows ion chromatograms from the analysis of plasma at baseline, and at 10 and 90 min after intravenous injection of [11C]PBR28 (710 MBq; A_m: 383.5 GBq/µmol) in one subject. The plasma matrix did not interfere with the ionization or detection of carrier PBR28 or of the internal standard, and no interfering peak was observed in the ion cha...
ograms. The measured ratios of carrier to internal standard peak area gave the true concentrations of carrier PBR28 from the linear calibration curve (Supplementary Fig. S4).

**LC–MS/MS measurement for AIF determination.** The $A_m$ values of $[^{11}C]$PBR28 doses administered to three different human subjects for PET imaging were measured with LC–MS/MS. These values were used to convert arterial plasma $[^{11}C]$PBR28 radioactivity from conventional AIF measurements at four timepoints into picomolar concentrations of carrier PBR28. This allowed the two datasets (radiometric and LC–MS/MS) to be compared. The plasma concentration of carrier PBR28 decreased as the $A_m$ value of the injected radiotracer increased, as would be expected for similar administered amounts of radioactivity per weight of subject. Plasma concentrations of carrier PBR28 calculated from radiometric measurement of $[^{11}C]$PBR28 radioactivity were higher than those measured with LC–MS/MS. After correcting for the systematic difference between measurements, the carrier PBR28 concentrations determined by radiometric and LC–MS/MS methods were highly comparable (Table 3), including those at very low levels of carrier concentration (~5 pM). Further experiments showed that the LC–MS/MS method had adequate sensitivity to measure $[^{11}C]$PBR28 in plasma at 23 timepoints for up to 90 min following intravenous injection of the radiotracer at different molar activities.

**Comparisons of AIFs for $[^{11}C]$PBR28 from LC–MS/MS and radiometric methods.** The carrier PBR28 concentrations in plasma samples measured with LC–MS/MS were transformed into radioactivity data using $A_m$ measured with LC–MS/MS. An example of a log-linear plot of plasma $[^{11}C]$PBR28 radioactivity versus time (i.e., AIF) from the new LC–MS/MS method and the conventional radiometric method for a human subject injected with $[^{11}C]$PBR28 at a moderately high $A_m$ value (i.e., with low carrier) is illustrated in Fig. 4a. In this example, and in seven other PET experiments with $[^{11}C]$PBR28, the log-linear plots of AIFs from LC–MS/MS ran nearly parallel below the AIFs obtained via the conventional radiometric method. After correcting the plasma radioactivity measured with the γ-counter for the systematic difference between the methods (as described above, see Supplementary Fig. S3), the AIF measured with LC–MS/MS became virtually superimposed with that measured radiometrically (Fig. 4b) for each example. AIF plots from radiometric and LC–MS/MS measurements for human subjects administered with $[^{11}C]$PBR28 of a low $A_m$ of 141.8 GBq/µmol and a high $A_m$ of 631.1 GBq/µmol are shown in Fig. 4c,d, respectively.

To examine the impact of different types of AIF measurement on the input function for kinetic modeling of PET data, we compared areas under the curve (AUCs) for the plasma time-activity curves. On average, AUC calculated from LC–MS/MS was 31% lower than that calculated with the radiometric method ($t = -11.9, p < 0.001$), and 8% lower than that calculated with the corrected radiometric method ($t = -6.0, p < 0.001$) (Table 4). VAR, Pearson correlation coefficient, and ICC, were respectively 37%, 0.97, and 0.07 for LC–MS/MS versus direct radiometric method, and 8%, 0.99, and 0.91 for LC–MS/MS versus the corrected radiometric method.

**Discussion**

This study assessed the feasibility of using LC–MS/MS for measuring AIF in human subjects undergoing PET scanning with a $[^{11}C]$-labeled radiotracer. LC–MS/MS analysis was found to provide a convenient and sensitive method for measuring the AIF of a $[^{11}C]$-labeled tracer without measuring its radioactivity. This method can be performed on multiple blood samples without the time and logistical constraints of the conventional radiometric method. The $A_m$ measured during the production of a radiotracer may be used to transform plasma carrier concentrations into AIF radioactivity data.

---

**Table 3.** Carrier PBR28 concentration (pM) in arterial plasma after $[^{11}C]$PBR28 injection. a Determined with LC–MS/MS for each radiometric and LC–MS/MS method. b From radiometric measurement before (A) and after (B) the correction of radioactivity (for the systematic difference between LC–MS/MS and γ-counter measures).
During the LC–MS/MS analysis of the triad, \([^{11}\text{C}]\), \([^{12}\text{C}]\), and \([^{13}\text{C}]\), in all four tested radiotracers, we observed a higher \([^{13}\text{C}]\) to \([^{12}\text{C}]\) ratio than in the respective non-radioactive standard (Table 1). The MS/MS technique demonstrated that 13C-enrichment had occurred in the carrier at the same position that had been labeled with carbon-11 during radiotracer synthesis. As clearly shown for \([^{11}\text{C}]\)PBR28, the degree of isotopic enrichment correlated with \(A_m\) value. Therefore, the augmented \([^{13}\text{C}]\) to \([^{12}\text{C}]\) ratio was not due to an isotope effect in the synthesis or purification of the radiotracer. The carbon-11 for labeling each radiotracer was produced by the \(14\text{N}(p,\alpha)^{11}\text{C}\) reaction on nitrogen with a 16 MeV beam of protons which degrade in energy on progressing through the gas target. An explanation for the \(A_m\)-related 13C-enrichment is the co-occurrence of the \(14\text{N}(p,2p)^{13}\text{C}\) reaction. For irradiations of nitrogen gas with 13.2 MeV protons, the \(14\text{N}(p,2p)^{13}\text{C}\) reaction has a total cross section of 74.2 mb (millibarn) which is very similar to that for the \(14\text{N}(p,\alpha)^{11}\text{C}\) reaction (68.9 mb)\(^{23}\). Therefore, the mass of generated carbon-13 is expected to be similar to the mass of carbon-11 produced. For a typical 40-min irradiation producing about 75 GBq of carbon-11, this amount would be about 10 nmol, or roughly enough to explain the 13C-enrichment seen for carrier in doses of \([^{11}\text{C}]\)PBR28.

The \(A_m\) value of a radiotracer needs to be determined accurately in order to derive mass of carrier from radioactivity or vice versa. A previous study from our laboratory reported an MS/MS technique for isolating \([^{11}\text{C}]\) to \([^{12}\text{C}]\) ratio that had been measured for the reference natural abundance ligand. In the present study, the

---

**Figure 4.** Examples of AIFs determined in human subjects with radiometric and LC–MS/MS methods for \([^{11}\text{C}]\)PBR28. (a) AIFs for one subject injected with \([^{11}\text{C}]\)PBR28 (10.1 MBq/kg; injected mass 92 pmol/kg, i.v.) with an \(A_m\) of 383.5 GBq/µmol. The AIF from radiometric method is without correction of γ-counter measured radioactivity for the systematic difference with LC–MS/MS. (b) Comparisons of AIFs from the same experiment after correction of γ-counter measured radioactivity. (c) AIFs from radiometric (corrected) and LC–MS/MS measurements in another human subject injected with \([^{11}\text{C}]\)PBR28 (10.7 MBq/kg; injected mass 229 pmol/kg, i.v.) at a lower \(A_m\) of 141.8 GBq/µmol. (d) AIFs from the same methods in a subject injected with radiotracer (9.5 MBq/kg; injected mass 44 pmol/kg, i.v.) at a higher \(A_m\) of 631.1 GBq/µmol. Note all data are for unchanged radiotracer alone (i.e., radiometabolites are excluded).
A\textsubscript{m} value for \([^{13}\text{C}]\)PBR28 was measured according to the same principle except that the specific \([^{11}\text{C}]\), to \([^{12}\text{C}]\) ratio of the carrier in the radiotracer was used for greater accuracy.

The sensitivity and dynamic range of MS/MS was found to be adequate to measure all three isotopologues in \([^{13}\text{C}]\)PBR28 with relatively high \(A_{m}\) values (~ 1000 GBq/µmol) (Supplementary Fig. S2). Such radiotracer preparations allowed injection of more diluted sample into the LC–MS/MS and thus measurement of carrier \([^{12}\text{C}]\) without saturating the detector. The \(A_{m}\) values determined from \([^{11}\text{C}]\), and \([^{13}\text{C}]\) as well as the \([^{12}\text{C}]\) to \([^{13}\text{C}]\) ratio, were found to be valid because direct measurement of the \([^{11}\text{C}]\), \([^{12}\text{C}]\), and \([^{13}\text{C}]\) triad yielded similar results (Table 2). The \(A_{m}\) value measured with a γ-counter was used to compare these two sets of data.

These findings further demonstrated that the LC–MS/MS technique can isolate and measure very low kBq levels of \([^{13}\text{C}]\) and, consequently, that this capability can be used to evaluate the accuracy of radioactivity measurements from radiation detectors such as ionization chambers and γ-counters. Building on this work, we compared the \(A_{m}\) values of \([^{13}\text{C}]\)PBR28 measured with LC–MS/MS with those determined using an ionization chamber and a γ-counter. The \(A_{m}\) (mean ± SD) value measured by ionization chamber was close to that obtained via LC–MS/MS, whereas that obtained via γ-counter was appreciably higher. Thus, comparing the three sets of \(A_{m}\) data revealed a significant difference in radioactivity estimates between the two commonly used radiometric methods.

Typically, an ionization chamber is calibrated for measuring carbon-11 with a pair of surrogate radioisotope standards, \([^{135}\text{Cs}\) \((t_{1/2} = 30.17 \text{ years}; \beta \text{-}\gamma 662 \text{ keV})\) and \([^{57}\text{Co}\) \((t_{1/2} = 271.79 \text{ days}; \beta \text{-}\gamma 122,136 \text{ keV})\), and a γ-counter with a different standard, \([^{68}\text{Ge}\) \((t_{1/2} = 270.8 \text{ days}; \text{decays to }^{68}\text{Ga}; t_{1/2} = 67.6 \text{ min}, \beta \text{-}\epsilon \gamma, \beta \text{-}\gamma\)). Clearly, these surrogate isotopes have decay modes that are very different from those of \([^{11}\text{C}]\) (\(\beta\text{-}\gamma, \sim 99.8\%\)). Moreover, the accuracy of radioactivity measured with an ionization chamber or γ-counter is well known to be influenced by sample volume and geometry effects. Studies seeking to measure positron-emitters in ionization chambers have been conducted with fluorine-18 \((t_{1/2} = 109.8 \text{ min})\), but none have used shorter-lived carbon-11. Indeed, we have previously observed in our facility that identical ionization detectors calibrated in the same way with the same surrogate standards can give estimates of carbon-11 radioactivity differing by up to 12%.

During the course of the present study, we thoroughly investigated whether such differences could be ascribed to detector dead-time and linearity, sample geometry, and volume effects, to the material of the sample container (glass or polypropylene), or to measurement time. None of these factors accounted for the observed differences. These results underscore the role that a sensitive MS/MS technique may play in checking radioactivity measured with radiometric techniques. Notably, the MS/MS technique obviates sample volume and geometry concerns.

| Human subject | AUCs (kBq × min/mL) | Radiometrica | Difference (%)b |
|--------------|---------------------|--------------|-----------------|
|              | LC–MS/MS A | B | A | B |
| 1            | 328        | 470 | 356 | 36 | 8 |
| 2            | 308        | 485 | 351 | 44 | 13 |
| 3            | 329        | 437 | 339 | 28 | 3 |
| 4            | 332        | 465 | 342 | 33 | 3 |
| 5            | 334        | 500 | 365 | 40 | 9 |
| 6            | 460        | 686 | 505 | 39 | 9 |
| 7            | 336        | 517 | 380 | 42 | 12 |
| 8            | 518        | 730 | 559 | 34 | 8 |
| Mean ± SD   | 368 ± 77   | 536 ± 109 | 399 ± 84 | 37 ± 5.3 | 8.1 ± 3.6 |
| RSD (%)      | 21         | 20  | 21  |     |      |

* Table 4. AUCs (kBq × min/mL) for the plasma time-activity curves from LC–MS/MS and radiometric measurements. aAUCs from radiometric measurements before (A) and after (B) the correction of radioactivity (for the systematic difference between LC–MS/MS and γ-counter measures). bBetween the AUC from LC–MS/MS and that from the radiometric method for AUC dataset A and AUC dataset B.
of 139 to 631 GBq/µmol. 13C-Labeled radiotracers are typically administered with molar activities at the lower end of this range. The entire range of quantification was achieved by injecting as little as 1/20th of each plasma sample onto the LC–MS/MS. If using a radiotracer with an exceptionally higher \( A_m \) value, quantification could still be achieved by increasing the injection volume, from for example 10 to 25 µL, or concentrating the plasma sample two-fold, although the LC procedure might consequently need some modification. In addition, it is expected that quantification limits would vary with the type of radiotracer carrier being measured.

With regards to measuring AIF for \( [13C]PBR28 \), when the radioactivity was corrected for the difference between radioactivity measured by y-counter and by LC–MS/MS, the plasma concentration curves from the two methods matched (Fig. 4). The difference likely occurred because LC–MS/MS performs absolute quantification of the carrier whereas the y-counter measures radioactivity relative to the surrogate radioisotope used for calibration. The radioactivity (Bq) is given by the product of the decay constant of the radionuclide and the number of un-decayed radioactive atoms. Thus, the LC–MS/MS measurement described here is expected to give absolute radioactivity, given that it is derived from the mass of \( [13C]_m \), the decay constant of carbon-11, and Avogadro’s number.

The AUCs for plasma time-activity curves from the LC–MS/MS method were 8.1 ± 3.6% \((n = 8)\) lower than those from the radiometric method with corrected radioactivity. Nonetheless, the %RSD of AUCs calculated for 8 subjects was the same for the two methods and showed good correlation \((\text{Pearson } r = 0.987; p = 0.01)\). Thus, the LC–MS/MS method is as reproducible as the radiometric method for measuring AIFs.

**Conclusion**

The LC–MS/MS of fast-decaying PET radiotracers provides interchangeable mass and radioactivity data and offers the convenience of measuring AIF through the carrier of the radiotracer instead of radioactivity. The method, here exemplified with \( [13C]PBR28 \), circumvents possible radiometabolite interference and error due to volume and geometry effects associated with radiometric measurements. Potentially, this non-radiometric method might allow measurement of AIF on stored plasma samples by analytical service laboratories that perform LC–MS/MS quantifications. In such instances, AIFs in radioactivity unit can be derived from the \( A_m \) measured during production of the radiotracer. Taken together, the LC–MS/MS method poses a convenient, non-radiometric, reproducible, and sensitive method for measuring AIF, deserving of widespread application in the expanding PET imaging field.

**Methods**

The Supplementary Methods describe: \( 1)\) Materials; \( 2)\) Radiosynthesis; \( 3)\) Measurement of \( A_m \) using HPLC and an ionization chamber; \( 4)\) Technical aspects of AIF measurement by literature radiometric method; \( 5)\) Preparation of PBR28 and \( [13C,2H_3]PBR28 \) internal standard (IS) stock solutions; \( 6)\) Extraction of carrier PBR28 and \( [13C,2H_3]PBR28 \) IS; \( 7)\) Recovery, matrix effect, stability, and reproducibility for LC–MS/MS quantification of carrier PBR28 in plasma.

**\( A_m \) of \([13C]PBR28 \) by LC–MS/MS.** An aliquot of a \( [13C]PBR28 \) preparation was diluted with LC mobile phase either 15-fold for measuring \( [13C] \) and \( [13C] \), or 40-fold for measuring \( [13C] \), \( [13C] \), and \( [13C] \). A sample (5 µL; \( n = 6 \) or 3) was injected onto LC–MS/MS (API 5000; Sciex; Redwood City, CA). Analysis was performed using LC method and MS/MS settings already described. A second transition, \( m/z \) 349 → 121, was included in the method requiring acquisition of \( [13C]_i \), \( [13C]_i \), and \( [13C]_i \). In \( A_m \) measurements based on acquisition of \( [13C]_i \) and \( [13C]_i \), the \( 13C \) peak area of the carrier was converted into the \( 12C \) peak area using the ratio \( [13C]_i/[12C]_i \), having a single natural abundance \( 1H \) or \( 17O \) atom. \( A_m \) was determined from the peak areas of radioactive and carrier species as \( (A^*)/(A + A^*) \times A_m \), where \( A^* \) is the sum of peak areas for \( [13C] \) and \( [12C] \), and for the calculated area for the same species containing carbon-13, \( A \) is the sum of the peak areas for \( [13C] \) and \( [12C] \), and \( \% \) is the theoretical carrier-free \( A_m \) of carbon-13 (3.413 × 10^20 Bq/mol, the product of ln2/\( n \) and Avogadro’s number).

**Ratio of \([13C] \) to \([12C] \).** The MS/MS instrument was tuned with reference ligands (PBR28, (R)-rolipram, DPA713, and (R)-PK11195). A method was set up to acquire \( [M + H]^+ \→ \) product ion transitions for \( [13C] \) and \( [13C] \), (which includes \( 13C \) species containing a single \( 1H \), \( 13N \), or \( 17O \) atom of natural abundance) for each carrier, as follows: PBR28, \( m/z \) 349/350 → 121/122; (R)-rolipram, \( m/z \) 276/277 → 191/192; DPA713, \( m/z \) 367/368 → 266/267 and (R)-PK11195, \( m/z \) 353/354 → 297/298 (Table 1). Radiotracer samples were analyzed after full radioactive decay. Specifically, the sample was diluted (100–500 fold) and injected (5 µL; \( n = 4 \)) onto the LC–MS/MS. Radiotracer’s carrier was chromatographed on a C18 column (2 × 20 mm, 3 µm; Phenomenex, Torrance, CA) using a similar water-acetonitrile (10 mM ammonium acetate or 0.2% acetic acid) gradient as previously reported. The ratio of peak areas for product ion from \( [13C] \), to that from \( [13C] \), multiplied by 100, gave the ratio \( [13C]_i/[12C]_i \), as a % value.

Reference PBR28, (R)-rolipram, DPA713, and (R)-PK11195 were analyzed similarly, and the ratio of \( [13C] \), and \( [13C] \), for each was determined for each.

**Radioactivity and carrier in \([13C]PBR28 \).** In each of three glass vials, IS solution (2 ng) in dimethylformamide (DMF; 1 mL) was mixed with \( [13C]PBR28 \) preparation (3–5 µL) and the radioactivity counted with a calibrated y-counter (model 1480 Wizard; Perkin-Elmer, Waltham, MA). After radioactivity decay, a 50 µL-aliquot of each sample was diluted with 450 µL of 1% acetic acid in acetonitrile (50% v/v), and a sample was injected (5 µL) onto LC–MS/MS. Samples prepared by mixing the IS solution with known concentrations of reference PBR28 (10–0.3125 ng/mL DMF) were analyzed similarly to provide a calibration curve. Except for
the LC column (3 μm; 2 × 50 mm; Phenomenex), the LC–MS/MS method used for the quantification of carrier PBR28 was the same as described for the plasma analysis (below).

**Arterial blood sampling from human subjects injected with [¹³C]PBR28.** Blood samples used in this study were drawn from human subjects who were recruited by the Molecular Imaging Branch of the National Institute of Mental Health (NIMH). The study was approved by the National Institutes of Health (NIH) Combined Neurosciences Institutional Review Board (NCT 01547780; NCT 01851356; NCT 02233868). The selected participants signed informed consent before entering the study. After [¹¹C]PBR28 injection, arterial blood samples were drawn at 15-s intervals up to 2 min and 30 s and thereafter at 3, 4, 6, 8, 10, 15, 20, 30, 40, 50, 60, 75 and 90 min. Before injection of the radiotracer, baseline arterial blood was drawn, centrifuged, and plasma used for a control LC–MS/MS (carrier-free) measurement. All methods were performed in accordance with the relevant guidelines and regulations of the NIH.

**AIF for [¹¹C]PBR28 by radiometric method.** Arterial blood samples were immediately centrifuged after withdrawal and 1.5–2.0 min of transportation. Samples in Eppendorf tubes (cap sealed with Parafilm M) were centrifuged at 1800 g after withdrawal and 1.5–2.0 min of transportation. Samples in Eppendorf tubes (cap sealed with Parafilm M) were centrifuged at 1800 g after withdrawal and 1.5–2.0 min of transportation. Samples in Eppendorf tubes (cap sealed with Parafilm M) were centrifuged at 1800 g after withdrawal and 1.5–2.0 min of transportation. Samples in Eppendorf tubes (cap sealed with Parafilm M) were centrifuged at 1800 g after withdrawal and 1.5–2.0 min of transportation. Samples in Eppendorf tubes (cap sealed with Parafilm M) were centrifuged at 1800 g after withdrawal and 1.5–2.0 min of transportation. To examine the impact of different types of AIF measurement on the input function for kinetic modeling of PET data, we calculated AUCs (by trapezoidal method) from the LC–MS/MS and radiometric methods (corrected and uncorrected). The resultant AUCs data for eight subjects and their mean ± SD and relative standard deviation (RSD (%)) were tabulated for each method. Similarities between LC–MS/MS and the radiometric methods were assessed with paired samples t test, test–retest variability (VAR, absolute difference between methods divided by their mean value), Pearson correlation coefficients, and intraclass correlation coefficients (ICC). The data were analyzed using IBM SPSS Statistics for Mac (version 26, copyright IBM Corporation 2019). P values less than 0.05 were considered statistically significant.

Received: 3 March 2020; Accepted: 18 September 2020
Published online: 15 October 2020

**References**

1. Phelps, M. E. Positron emission tomography provides molecular imaging of biological processes. *Proc. Natl. Acad. Sci. USA* **97**, 9226–9233 (2000).
2. Chua, S. & Groves, A. Biomedical positron emission tomography (PET) imaging. In *Biomedical Imaging: Applications and Advances. Woodhead Publishing Series in Biomedicals* (ed. Morris, P.) 3–40 (Elsevier, Amsterdam, 2014).
3. McCluskey, S. P., Plisson, C., Rabiner, E. A. & Howes, O. Advances in CNS PET: state of the art for new imaging targets for pathophysiology and drug development. *Eur. J. Nucl. Med. Mol. Imaging* https://doi.org/10.1007/s00259-019-04488-0 (2019).
4. Bar-Shalom, R., Valdivia, A. Y. & Blaufox, M. D. PET imaging in oncology. *Semin. Nucl. Med.* **30**, 150–185 (2000).
5. Pike, V. W. Considerations in the development of reversibly binding PET radioligands for brain imaging. *Curr. Med. Chem.* **23**, 1818–1869 (2016).
6. Heurling, K. et al. Quantitative positron emission tomography in brain research. *Brain Res.* **1670**, 220–234 (2017).
7. Silfstein, M. & Abi-Dargham, A. Recent developments in molecular brain imaging of neuropsychiatric disorders. *Sem. Nucl. Med.* **47**, 54–63 (2017).
8. Herholz, K. & Heiss, W.-D. Positron emission tomography in clinical neurology. *Mol. Imaging Biol.* **6**, 239–269 (2004).
9. Vila-Costas, E., Vila-Costas, E., Vila-Costas, E., Vila-Costas, E., Vila-Costas, E. Imaging tau and amyloid-β proteinopathies in Alzheimer disease and other conditions. *Nat. Rev. Neurosurg.* **14**, 225–236 (2018).
10. Volkow, N. D., Fowler, J. S. & Wang, G.-J. Positron emission tomography and single-photon emission computed tomography in substance abuse research. *Semin. Nucl. Med.* **33**, 114–128 (2003).
11. Matthews, P. M., Rabiner, E. A., Passchier, I. & Gunn, R. N. Positron emission tomography molecular imaging for drug development. *Br. J. Clin. Pharmacol.* **73**, 175–186 (2011).
12. Tonietto, M. et al. Plasma radiometabolite correction in dynamic PET studies: insights on the available modeling approaches. J. Cerebr. Blood Flow Metab. 36, 326–339 (2016).
13. Zimmerman, B. E. & Cessna, J. T. Development of a traceable calibration methodology for solid 68Ge/68Ga sources used as a calibration surrogate for 18F in radionuclide activity calibrators. J. Nucl. Med. 51, 448–453 (2010).
14. Lodge, M. A., Holt, D. P., Kinahan, P. E., Wong, D. F. & Wahl, R. L. Performance assessment of a NaI(Tl) gamma counter for PET applications with methods for improved quantitative accuracy and greater standardization. EJNMMI Phys. 2, 11 (2015).
15. Shetty, H. U., Morse, C. L., Zhang, Y. & Pike, V. W. Characterization of fast-decaying PET radiotracer solvents through LC–MS/MS of constituent radioisotopes. EJNMMI Res. 3, 3 (2013).
16. Coplen, T. B. et al. Isotope-abundance variations of selected elements (IUPAC technical report). Pure Appl. Chem. 74, 1987–2017 (2002).
17. Bariard, E. et al. Synthesis and evaluation in monkey of two sensitive 13C-labeled arylxanilide ligands for imaging brain peripheral benzodiazepine receptors in vivo. J. Med. Chem. 54, 17–30 (2008).
18. Fujita, M. et al. Comparison of four [14C]labeled PET ligands to quantify translocator protein 18 kDa (TSPO) in human brain: ([1]P11195, PBR28, DPA-713, and ER176—based on recent publications that measured specific-to-nondisplaceable ratios. EJNMMI Res. 7, 84 (2017).
19. Selleri, S. et al. 2-Arylpyrazolo[1,5-a]pyrimidin-3-yl acetamides. New potent and selective peripheral benzodiazepine receptor ligands. Bioorg. Med. Chem. 9, 2661–2671 (2001).
20. Zanotti-Fregonara, P. et al. Kinetic analysis in human brain of [11C]-(R)-rolipram, a positron emission tomographic radioligand to image phosphodiesterase 4: a retest study and use of an image-derived input function. NeuroImage 54, 1903–1909 (2011).
21. Christman, D. R., Finn, R. D., Karlström, K. I. & Wolf, A. P. The production of ultra high activity [14C]-labeled hydrogen cyanide, carbon dioxide, carbon monoxide and methane via the 14N(p, α)11C reaction (XV). Int. J. Appl. Radiat. Isot. 26, 435–442 (1975).
22. Gomez-Vallejo, V., Gaja, V., Koziorowski, J. & Llop, J. Specific activity of 11C-labeled radiotracers: a big challenge for PET chemists. Positron Emission Tomography—Current Clinical and Research Aspects (ed. Hsieh, C.-H.) 183–210 (InTech, Rijeka, 2016).
23. MaLeod, A. M. & Reid, J. M. Proton nuclear reaction cross sections in nitrogen at 13 MeV. Proc. Phys. Soc. 87, 437–444 (1966).
24. Zimmerman, B. E., Kubiecz, G. J., Cessna, J. T., Plascak, P. S. & Eckelman, W. C. Radioassays and experimental evaluation of dose calibrator settings for 18F. Appl. Radiat. Isot. 54, 113–122 (2001).
25. Cessna, J. T., Schultz, M. K., Leslie, T. & Bores, N. Radionuclide calibrator measurements of 18F in a 3 ml plastic syringe. Appl. Radiat. Isot. 66, 988–993 (2008).
26. Zoghbi, S. S. et al. PET imaging of the dopamine transporter with 18F-FECNT: a polar radiometabolite confounds brain radioligand measurements. J. Nucl. Med. 47, 520–527 (2006).

Acknowledgements
We thank Drs. F.G. Siméon and C. Brouwer for the synthesis of [13C,2H3]PBR28 and DPA713, respectively, Drs. J. Hong and S. Tehu for the radiosynthesis of [14C]PBR28, and the NIH Clinical PET Department (Chief, Dr. P. Hirschovitch) for carbon-11 production. This study was funded by the Intramural Research Program of the National Institute of Mental Health, National Institutes of Health (IRP-NIMH-NIH: ZIA-MH002793; ZIA-MH002795-13).

Author contributions
H.U.S., S.S.Z., and V.W.P. designed the study; H.U.S., S.S.Z., C.L.M., and A.K. performed the research; J.H. and R.B.I. contributed to the data analysis; H.U.S. and V.W.P. wrote the paper. All authors reviewed manuscript content.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-73646-4.

Correspondence and requests for materials should be addressed to H.U.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2020.