Rapid and accurate $^{13}$CO$_2$ isotopic measurement in whole blood: comparison with expired gas

MARTIAL DANGIN, 1,2 JEAN CLAUDE DESPORT, 1 PIERRE GACHON, 1 AND BERNARD BEAUFRE`RE 1

1Laboratoire de Nutrition Humaine, Université Clermont Auvergne, Centre de Recherche en Nutrition Humaine, 63009 Clermont-Ferrand, Cedex 1, France; and 2Nestlé Research Center, CH-100 Lausanne 26, Switzerland

Dangin, Martial, Jean Claude Desport, Pierre Gachon, and Bernard Beaufrère. Rapid and accurate $^{13}$CO$_2$ isotopic measurement in whole blood: comparison with expired gas. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E212–E216, 1999.—Determination of $^{13}$CO$_2$ enrichment on the CO$_2$ released from blood by acid has been used in situations in which breath sampling is difficult. This method can be improved by measuring this enrichment on the CO$_2$ spontaneously released from blood. Therefore, simultaneous comparisons of $^{13}$CO$_2$ content between breath and arterialized blood added with or without acid were performed in 51 samples from human studies, using the statistical method of Bland and Altman (J. M. Bland and D. G. Altman. Lancet 1: 307–310, 1986). Strong relationships exist between the methods ($r > 0.99$) expressed in atom percent excess (APE). Compared with breath, the acid method overestimates the $^{13}$CO$_2$ enrichment ($0.318 \pm 0.632$ APE $\times 1000$, $P < 0.001$). The acid-free method shows similar enrichments to breath ($0.003 \pm 0.522$ APE $\times 1000$, $P = 0.97$) with good precision and degree of agreement (95% confidence interval $0.15$ APE $\times 1000$). The analysis can be performed up to 5 days after sampling with a good reproducibility. In conclusion, measuring $^{13}$CO$_2$ enrichments on the CO$_2$ spontaneously released from blood is feasible, gives identical results to the breath method, and lessens operator manipulations. It allows study of situations in which the breath sampling method is not feasible.

The measurements of $^{13}$CO$_2$ enrichment are required in numerous studies to estimate either nutrient oxidation rates (17), CO$_2$ production (9), or assimilation of various $^{13}$C-labeled substrates (7; i.e., breath tests). For these purposes, $^{13}$CO$_2$ atom percent (AP) is usually determined by isotope ratio mass spectrometry on breath samples collected at sequential time points (17). However, voluntary breath sampling can be difficult or impossible to perform in various situations such as ventilatory abnormalities, artificially ventilated patients, or in specific populations such as newborns or patients with neurological disorders (8, 14). Thus two alternatives have been proposed to solve this problem. The first one is to sample gas from the ventilated hood of an indirect calorimeter (8). This method is not widely used, in particular because large volumes of gas are needed for isotope ratio mass spectrometry analysis due to atmospheric air dilution. In addition, this technique is limited by the mandatory utilization of the indirect calorimetry system, which is not required for breath test or for the estimation of CO$_2$ production by an isotopic dilution method. The second method (4, 6, 10, 14, 15) analyzes the gaseous CO$_2$ released from blood by acid addition. Compared with the breath method, this method is more demanding due to operator manipulations. For diagnosis purposes, it is important that blood samples can be processed rapidly, reliably, and with minimal operator manipulations. Considering that 1) blood CO$_2$ is spontaneously released in the air and 2) the sensitivity of the isotope ratio mass spectrometer has dramatically increased over the past few years, it should be possible to improve the latter method by the measurement of $^{13}$CO$_2$ AP spontaneously released from blood (e.g., without acid addition). Thus the aim of the present work was to determine whether CO$_2$ released from the blood without acid addition can be used to measure $^{13}$CO$_2$ AP. The technical aspects of this methodology were validated, and simultaneous comparisons for breath, blood with acid, and blood without acid were made at each sampling point.

SUBJECTS AND METHODS

Subjects and experimental protocols. Blood and gas samples were obtained from nine young healthy subjects (age 30.6 ± 11.7 yr, weight 63.9 ± 5.4 kg, height 171 ± 7 cm, mean ± SD) who participated in two different protocols that were previously described in detail (2, 11). The two protocols were approved by the ethical committee of Clermont-Ferrand. Briefly, five subjects received (11) a primed continuous constant intravenous infusion of L-[1-13C]leucine for 10 h after a prime dose of [13C]bicarbonate, and samples were collected before the tracer infusion and when the $^{13}$CO$_2$ AP had reached a plateau (510, 540, 580, and 600 min). The four other volunteers (2) received a single oral load of 30 g of whey protein added with free L-[1-13C]leucine (13 mmol/kg). Samples were taken before and...
after (at 60, 120, 180, 240, and 300 min) the administration of oral tracer and were utilized for the present study. We chose this latter study because the obtained $^{13}$CO$_2$ AP both covered and exceeded the normal range usually observed in classic oxidation studies at steady state. A total number of 51 time points were obtained. For each of these, expired gas and arterialized blood samples were collected simultaneously. Breath samples were collected as previously described (6). Before blood sampling, the hand was warmed for 15 min at 60°C in a heated ventilated box as previously described (2). Immediately after sampling, the gas was injected from each sample (1 rotation/min) during 2 h at room temperature before the isotope analysis. Measurements were performed on the following three sets of samples: breath, CO$_2$ spontaneously released by blood, and CO$_2$ released from blood by lactic acid. Each method is further referred to as “Breath,” “Blood,” and “Blood+LA,” respectively.

Analytical methods and calculations. The $^{13}$CO$_2$ analyses were performed by gas chromatography isotope ratio mass spectrometry (μGas System; Fisons Instruments, VG Iotech, Middlewich, UK). Briefly, a sample (40 µl) of the gaseous content of each tube analyzed was automatically injected (Gilson automatic sampler) in the gas chromatograph (Hewlett-Packard 5890; Palo Alto, CA) in which the CO$_2$ was separated from the other components using a packed column (Chrompack column, type HAYSEP Q, 2.5 m × 1/8, 60/80 mesh; Les Ulis, France) at 100°C. The CO$_2$ was then introduced in the isotope ratio mass spectrometer to measure the $^{13}$C-to-$^{12}$C isotope ratio of the sample (RSAM) versus a reference gas (RREF). The $^{13}$C-to-$^{12}$C ratio of the samples was expressed as delta per mil (‰) versus RREF after Craig corrections (REF; see Ref. 3)

$$\delta^{13}C_{VS.REF} = \frac{R_{SAM} - R_{REF}}{R_{REF}} \times 1,000$$

In this study, the reference gas had a $\delta^{13}$C of $-31.00$ relative to the international standard reference Pee Dee Belemnite ($R_{PDB} = -0.0112372$). This calibration was obtained by periodic comparison with other laboratories.

The results obtained in these conditions were corrected to express the values in δ‰ vs. PDB. The values calculated were then converted in AP using the following formula

$$AP = \frac{\left(\frac{\delta^{13}C}{1,000} + 1\right) \times R_{PDB}}{\left(\frac{\delta^{13}C}{1,000} + 1\right) \times R_{PDB} + 1} \times 100$$

Finally, the $^{13}$CO$_2$ atom percent excess (APE) was obtained by subtracting the background AP (before tracer administration) from the sample AP (during or after tracer administration). Results obtained were expressed in δ‰ vs. PDB, AP, and in APE × 1,000.

Statistical analysis. Comparison between the methods was tested using the statistical method described by Bland and Altman (1). The statistics (Student’s t-test, ANOVA) were performed using Statview 4.02 from Abacus Concepts (Berkeley, CA). Results were expressed as means ± SD.

RESULTS

Interassay and intra-assay reproducibilities and storage. Interassay and intra-assay reproducibilities of the measurement on Breath, Blood, and Blood+LA were tested as shown in Table 1. The area of the CO$_2$ peak derived from arterialized venous blood decreased by 60% compared with Breath or Blood+LA. Despite this difference, the SD of Blood in the interassay replicate (n = 5 subjects) was similar to Breath, whereas the SD of Blood+LA was slightly higher. Intra-assay reproducibility was good for all three methods, indicating that up to five replicates can be performed in the same tube. There was no statistical difference between the Breath and the Blood methods. In contrast, Blood+LA was significantly higher than Breath (P < 0.001, t-test). When 1.5 ml of lactic acid were analyzed, traces of CO$_2$ were detected, but the very low intensity of the peak did not allow any measurement of the $^{13}$C isotopic content.

The storage of Blood samples at −20°C for 5 days was also tested in another batch of blood samples. No significant change in the $^{13}$CO$_2$ isotopic ratio was found (P = 0.69, t-test) between Blood immediately treated (−20.28 ± 0.08 δ‰ vs. PDB) and Blood treated after 5 days of storage (−20.30 ± 0.08 δ‰ vs. PDB). In addition, the storage did not modify the reproducibility of the isotopic analysis (SD = 0.08 δ‰ vs. PDB in each case, n = 5 subjects).

Relationship, precision, and agreement between the methods for $^{13}$CO$_2$ isotopic content. The $^{13}$C-to-$^{12}$C isotope ratio tested in Breath ranged from −25.66 to −6.05 δ‰ vs. PDB, in Blood from −26.75 to −7.49 δ‰ and in Blood+LA from −21.5 to −2.79 δ‰ vs. PDB.

As shown in Fig. 1, there were strong positive relationships between Breath and Blood and between Breath and Blood+LA expressed in AP (r > 0.99). In the range of our values, Blood+LA was systematically higher than Breath and Blood. Moreover, in both cases, y-intercepts were not significantly different from zero (0.30 < P < 0.49, ANOVA).

With the use of the Bland and Altman (1) plot (difference between the 2 methods of measurement vs. their mean at each time point), there was good precision and agreement between the AP of Breath and AP of Blood $^{13}$CO$_2$ isotopic ratios, as shown in Fig. 2. Indeed, there was no statistical difference between the two methods (0.10 ± 0.11 AP × 1,000, P = 0.31, t-test for

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* Significant difference (P < 0.05, t-test) between interassay Breath and the other interassay methods.
difference) and no relationship between the difference and the mean \( (P = 0.56, \text{ANOVA}) \). However, the scatter of the difference increased as \( ^{13}\text{CO}_2 \) enrichment increased. The 95% confidence interval ranged from \(-0.099 \) to \(0.100 \) AP \( \times 1,000 \).

In contrast, the difference between AP of \( ^{13}\text{CO}_2 \) in Breath and AP of Blood was always positive (Fig. 2) and was statistically different from zero \( (5.86 \pm 0.66 \text{ AP} \times 1,000, P < 0.001) \). In addition, despite the absence of a relationship between the difference against their mean \( (P = 0.16) \), the scatter of the difference also increased as the \( ^{13}\text{CO}_2 \) abundance increased. The 95% confidence interval was always positive despite the precision (range from 5.678 to 6.049 AP \( \times 1,000 \)).

Relationship, precision, and agreement between the methods for \( ^{13}\text{CO}_2 \) isotopic enrichments. Regression line equations for APE of Breath vs. APE of Blood and APE of Breath versus APE of Blood + LA are given in Fig. 3. The slope for APE of Breath versus APE of Blood \( (1.0003 \pm 0.013, 0.2 < P < 0.1) \) and the y-intercept \( (-0.015 \pm 0.110 \text{ APE} \times 1,000, P = 0.89) \), statistically, did not differ from the median slope and zero, respectively. In contrast, for APE of Breath versus APE of Blood + LA, the slope was different from the median slope \( (1.029 \pm 0.015, P < 0.001) \), whereas the y-intercept was not different from zero \( (0.131 \pm 0.129 \text{ APE} \times 1,000, P = 0.31) \).

The difference between APE of Breath and APE of Blood \( (0.003 \pm 0.522 \text{ APE} \times 1,000) \) did not appear to be biased \( (P = 0.97, t\text{-test of the difference}) \), and there was no relationship between the difference vs. their mean \( (P = 0.60) \). Despite this observation, the difference increased as the \( ^{13}\text{CO}_2 \) enrichments increased. However, the 95% confidence interval \((-0.144 \text{ to 0.150 APE} \times 1,000) \) was small enough to ensure that the method based on the free \( ^{13}\text{CO}_2 \) released from blood can be used with an acceptable precision and degree of agreement.

In contrast (Fig. 4B), the difference between APE of Breath and APE of Blood \( (0.318 \pm 0.632 \text{ APE} \times 1,000, P < 0.001; 95\% \text{ confidence interval } 0.141 \text{ to 0.496 APE} \times 1,000) \) is significantly different from zero. In addition, there was a positive statistical relationship between the difference \( (\text{APE of Breath} \times \text{APE of Blood}) \) and Breath vs. their mean \( (P = 0.02) \), indicating a slight overestimation of the enrichments at higher values.

**DISCUSSION**

Our intent was to develop an automated technique for measuring \( ^{13}\text{CO}_2 \) APE in conditions in which the

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**Fig. 2.** Difference between \( ^{13}\text{CO}_2 \) in Breath (\( \odot \)) or Breath + LA (\( \bullet \)) and Breath expressed in AP vs. their means. Dotted lines show means of the difference for Breath and Breath (0.10 AP \( \times 1,000 \)) and the difference between Breath + LA and Breath (5.86 AP \( \times 1,000 \)).

**Fig. 3.** Relationships between Blood and Breath (\( \odot \)) or Breath + LA and Breath (\( \bullet \)) expressed as \( ^{13}\text{CO}_2 \) atom percent excess (APE) \( = 1.03x + 0.015, r = 0.996; \text{Breath} + \text{LA} \times \text{APE} = 1.0297x + 0.131, r = 0.995 \).
breath method is difficult or impossible to perform. For this purpose, we have compared the results obtained by our method with the analysis performed in expired gas (considered as the reference method) and in gaseous CO2 released from blood by acid addition (4, 6, 10, 14, 15).

Comparison between exhaled air and gaseous CO2 released from blood by acid revealed higher 13CO2 APE in whole blood, which is in close agreement with previously published works (10, 14). Because lactic acid solution per se is not a significant provider of CO2, this difference cannot be due to lactic acid itself. Therefore, it implies that there is an isotopic fractionation during the reaction leading to CO2 production. This reaction is the following

\[
\text{CO}_2^{\text{gas}} \Leftrightarrow \text{CO}_2^{\text{dissolved}}
\]

\[
\text{CO}_2^{\text{dissolved}} + \text{H}_2\text{O} \Leftrightarrow \text{H}^+ + \text{HCO}_3^-
\]

the latter step being catalyzed in vivo by carbonic anhydrase. When lactic acid is added to a test tube containing blood, the equilibrium of the reaction is modified to produce gaseous CO2, and the relative contribution of carbonic anhydrase is strongly reduced. Therefore, the 13C content measured on the released CO2 primarily reflects the 13C content of bicarbonate. An isotopic effect was demonstrated when acid was added to dissolved bicarbonate, without carbonic anhydrase (5, 12, 13). The authors found that the 13C content of bicarbonate was higher compared with CO2, which is consistent with our results. A second isotopic effect was also shown at the carbonic anhydrase step (13). Indeed, in an in vitro system, enzymatic dehydration of bicarbonate dissolved in water results in an additive lower 13C content in the evolved CO2 compared with the bicarbonate, which is also in agreement with our findings. Therefore, the difference of 13C content that we observed between expired CO2 and CO2 derived from bicarbonate is likely to be due to these two factors.

Our data do not allow evaluation of their relative contribution. In this respect, it would be of interest to compare 13C content in expired CO2, whole blood, and plasma (i.e., without carbonic anhydrase) or to use carbonic anhydrase inhibitors.

When the 13CO2 content of exhaled air and the CO2 provided by acid addition were corrected by subtraction of their respective basal value, we found a modest but persistent overestimation of the enrichment, which increases as the 13CO2 content increases, in blood degassed by acid. Such results were also reported by Read et al. (14) but not by Denne and Kalhan (4) and El-Khoury et al. (6). However, in these studies, the statistical analyses were only performed on a limited number of samples. This uncertainty thus restricts the use of this method because such a technique has to give similar results to the measurement in breath, considered as the reference method.

In contrast, the measurements of the 13CO2 isotopic content spontaneously released by blood gave good precision and agreement with similar results to exhaled air whether it was expressed in AP or in APE. An isotopic fractionation may also exist at the alveolar blood-breath interface (i.e., in vivo) or at the water-air interface (i.e., in the test tube; see Ref. 16). The absence of difference in the carbon isotope analysis between the expired CO2 and the CO2 spontaneously released by blood suggests that the fractionation is negligible or similar under these two circumstances.

A potential analytical drawback of the method developed is the small quantity of CO2 dissolved in the blood and therefore evolved in the tube compared with the two other methods (Breath and Blood+LA). This could limit the number of replicates of isotope analysis in the same tube. Nevertheless, in our conditions, up to six replicates can be done with an excellent intra-assay reproducibility. In practice, duplicate or triplicate measurements give reliable results. In addition, the gas chromatography isotope ratio mass spectrometry analysis can be performed up to 5 days after blood sampling. Compared with the acid method, our technique is also superior for manipulator ease of performance and analysis. It simply requires 1 ml of arterialized whole blood introduced in a tube previously flushed with helium and agitated (1 rotation/min) during 2 h at room temperature before the isotope analysis.

Such a technique can be employed to estimate whole body nutrient oxidation rate by measuring separately 13CO2 APE in whole blood and CO2 production rate by indirect calorimetry in the situations mentioned in the Introduction. Furthermore, when indirect calorimetry
is unavailable, it should be possible to assess whole blood flow and blood CO2 concentrations and 13C enrichments. Finally, applications also exist to measure nutrient oxidation rate in specific organs and to quantify CO2 production rate.

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Address for reprint requests: B. Beaufre, Laboratoire de Nutrition Humaine, B.P. 321–58, rue Montalembert, 63009 Clermont-Ferrand Cedex 1, France.

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