New Strategy for in Vitro Determination of Carbonic Anhydrase Activity from Analysis of Oxygen-18 Isotopes of CO_2

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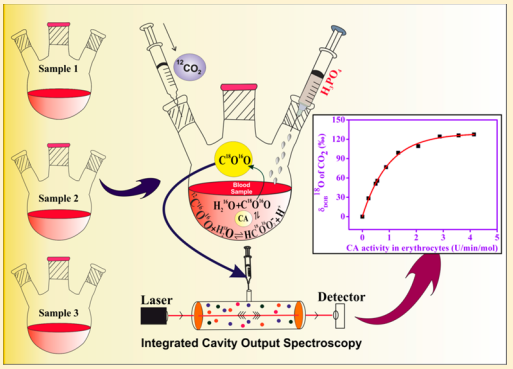
ABSTRACT: The oxygen-18 isotopic (^{18}O) composition in CO_2 provides an important insight into the variation of rate in isotopic fractionation reaction regulated by carbonic anydrase (CA) metalloenzyme. This work aims to employ an ^{18}O-isotope ratio-based analytical method for quantitative estimation of CA activity in erythrocytes for clinical testing purposes. Here, a new method has been developed that contains the measurements of $^{18}O/^{16}O$ isotope ratios during oxygen-18 isotopic exchange between $^{12}C^{16}O^{16}O$ and $H_2{}^{18}O$ of an in vitro biochemical reaction controlled by erythrocytes CA and estimation of enzymatic activity of CA from the isotopic composition of CO_2. We studied the enrichments of ^{18}O-isotope of CO_2 with increments of CA activities during isotopic fractionation reaction. To check the influence of subject-specific body temperature, pH, $H_2{}^{18}O$, and cellular produced CO_2 on this reaction, we performed the in vitro experiments in closed containers with variations of those parameters. Finally, we mimicked the exchange reaction at 5% [CO_2], 5‰ [$H_2{}^{18}O$], pH of 7.4, and temperature of 37 °C to create the physiological environment equivalent to that of the human body and monitored the exchange kinetics with variations of CA activities, and subsequently, we derived the quantitative relation between the ^{18}O-isotope of CO_2 and CA activity in erythrocytes. This assay may be applicable for rapid and simple quantification of carbonic anhydrase activity which is very important to prevent the carbonic-anhydrase-associated disorders in human.



Carbonic anhydrase (CA), a ubiquitous metalloenzyme that catalyzes the reversible hydration of CO_2 and water (H_2O) to form bicarbonate (HCO_3^-), is widely distributed in all living organisms, plants, and algal species.[1,2] There are five different CA families (α, β, γ, δ, and ζ) among which α-CAs are localized in humans. There have been 16 isoforms of α-CAs (CA I to CA VI, CA IX, CA XI, CA XII, CA XIII, CA XIV, and CA XV) isolated so far in which Zn(II) is the active site of the enzyme that coordinates the three histidine residues. These isoenzymes play an important role in regulating the physiological and pathophysiological functions in the body. During the past few decades, CA activity has been studied by both pharmacologists and physiologists.[3] Cytosolic CA II exhibits high activity and is widely distributed in red blood cells (erythrocytes). Although CA I presents 5−6 times higher activity than CA II, it shows only 15% of the activity as compared to that of CA II, and it is responsible for 50% of total CA activity in erythrocytes.[4,5] However, previous studies reported[6−8] that the changes of CA activity are associated with numerous diseases including edema, glaucoma, osteoporosis, and neurological disorders, where the catalytic activity of CA has been studied. Considerable data is now available to confirm the potential role of CA during cell growth in renal cancer, cervical cancer, and lung cancer. There is also interesting evidence that the prognostic value of carbonic anhydrase expression may be an important predictor of survival for renal cell carcinoma.[9,10] Therefore, there has been a growing interest in developing a simple assay method for quantitative estimation of CA activity. Although the traditional method provides useful information about the enzymatic assay of CA, the practical application of this method is limited due to a tedious and expensive process including blood sample collection, prolonged time for lab processing, and subsequent analysis by suitable spectroscopic technique. The assay is based on the spectrophotometric measurement of *para*-nitrophenol from hydrolysis of *para*-nitrophenyl acetate in the presence and absence of a specific inhibitor of CA.[11,12] However, the barriers to effective utilization of this method are the necessity of standardization from the knowledge of cell counts, maintaining the medium temperature throughout the process and overall processing of blood samples for prolonged time, suggesting that an alternate approach is desperately needed to overcome the above issues.

Early studies suggest[13−17] that oxygen-16 isotope (^{16}O) and oxygen-18 isotope (^{18}O) are rapidly exchanged between CO_2 and body ^{18}O-water catalyzed by CA to produce the $^{12}C^{18}O^{16}O$ isotope:

$$^{12}C^{16}O^{16}O + H_2{}^{18}O \xrightarrow{\text{carbonic anhydrase}} {}^{12}C^{18}O^{16}O + H_2{}^{16}O$$

This isotopic exchange during the physiological process has a large impact on isotopic composition of carbon dioxide in the human body. The ^{18}O-isotope may provide useful information for the estimation of erythrocyte CA activity as both the reactants and products diffuse rapidly across the cell membrane. Therefore, this isotopic fractionation suggests that there is a possibility to noninvasively estimate the CA activity in erythrocytes from monitoring of oxygen-18 isotopes of CO_2. However, no study to date has reported any method to exploit the isotopic exchange phenomenon to determine the CA activity in human body. In this study, we have explored a new method which can quantitatively estimate the CA activity from the analysis of oxygen-18 isotopes of CO_2.

We performed the in vitro study to monitor the generation of $^{12}C^{18}O^{16}O$ isotopes caused by the isotopic fractionation between $^{12}C^{16}O^{16}O$ and $H_2{}^{18}O$, and the experiments were designed in such a way that these would allow us to mimic the physiological environment of the human body. During the equilibrium of the reaction, the oxygen isotope of CO_2 is enriched with ^{18}O-isotope, and this exchange is regulated by CA. However, the enzymatic activity of CA is known to be sensitive to subject-specific body temperature, pH, intracellular CO_2, and ^{18}O of body H_2O. All of these factors may alter the exchange kinetics of the isotopic reaction resulting in variation of $^{12}C^{18}O^{16}O$ isotopic compositions of CO_2 in medium.

EXPERIMENTAL ANALYSIS

Blood Sample Preparation. Venous blood samples (10 mL) were collected from each participant in EDTA vacutainer tubes. The blood samples were allowed to centrifuge at 2000 rpm for 15 min, and the plasma was separated. The buffy coat was removed from the sample. Then, the RBC was washed with 0.9% NaCl solution, and it was allowed to spin at 4000 rpm for a few minutes. The erythrocyte packs were collected and lysed with ice-cold water. The ghost cells from the hemolysate solution were removed after centrifuging it at 10 000 rpm for 30 min. Carbonic anhydrase activity was determined from the fresh supernatant solution.

Integrated Cavity Output Spectrometer. A laser-based high-resolution integrated cavity output spectrometer (CCIA 36-EP, Los Gatos Research) was used to estimate the carbon dioxide and its isotopes ($^{12}C^{16}O^{16}O$ and $^{12}C^{16}O^{18}O$). The working details of ICOS have been described somewhere else.[18,19] The present ICOS system consists of a high-finesse optical cavity (~59 cm long) with two high-reflectivity mirrors ($R \sim 99.98\%$) at the two ends. The laser frequency was scanned over 20 GHz to record the absorption spectra of $^{12}C^{18}O^{16}O$ and $^{12}C^{16}O^{16}O$ at the wave numbers 4874.178 and 4874.448 cm^{-1} in the (2, 0°, 1) ← (0, 0°, 0) vibrational combination band of the CO_2 molecule. The enrichments of $^{12}C^{18}O^{16}O$ have been expressed by the conventional notation, $\delta^{18}O‰$ relative to the standard Pee Dee Belemnite (PDB). It is described as

$$\delta^{18}O‰ = \left[\frac{\left(\frac{^{18}O}{^{16}O}\right)_{sample}}{\left(\frac{^{18}O}{^{16}O}\right)_{standard}} - 1\right] \times 1000$$

$$\delta_{DOB}{}^{18}O‰ = (\delta^{18}O‰)_{sample} - (\delta^{18}O‰)_{blank}$$

where $(^{18}O/^{16}O)_{standard}$ is the international standard Vienna Pee Dee Belemnite value, i.e., 0.002 0672.

Carbonic Anhydrase Activity Measurement. Carbonic anhydrase activity was measured spectrophotometrically by following Armstrong et al.[20] with the modification described by Parui et al.[11] The hydrolysis rate of *p*-nitrophenyl acetate (PNPA) to *p*-nitrophenol gives the enzymatic activity of carbonic anhydrase. A specific inhibitor of CA, acetazolamide (AZM), was used to suppress the enzymatic activity of CA. The assay method is composed of a 1 cm cuvette containing 100 μL of hemolysate, 1.86 mL of tris buffer, and 20 μL of PNPA. The absorbance was measured by a UV−vis spectrophotometer (Shimadzu UV-2600 spectrophotometer) at 348 nm over the period of 3 min. One unit of enzyme activity was expressed as μmol of *p*-nitrophenol relased/min/μL from hemolysate at room temperature. The following formula was used to calculate total erythrocyte CA activity:

$$\text{CA activity} = \frac{A_3 - A_0}{5000} \times \frac{1}{3} \times \frac{2000}{5} \times 1000\ \mu\text{mol/min/mL}$$

where A_3 is the absorbance after 3 min, A_0 is the absorbance at 0 min, 5×10^3 M^{-1} cm^{-1} is the molar absorptivity of *p*-nitrophenol. The activity was normalized to 4.5×10^9 cells/mL.

Experimental Details. In this study, the whole reactions were carried out in sealed round-bottomed flasks. The flasks were tightly fitted with septum and adaptors. The adaptors were sealed with proper fittings. To minimize the effect of other gases within the flasks, all flasks were carefully purged with pure nitrogen gas. A 5 mL portion of hemolysate along with 5‰ $H_2{}^{18}O$ were placed in the flasks. Acetazolamide, the carbonic anhydrase inhibitor, was added into the flasks at desired quantities to prepare a wide variety of hemolysate solutions with various carbonic anhydrase activities. The flasks were kept for 2 h after addition of CO_2 gas to attain equilibrium. After 2 h of the reaction, the acidification of the solution was done by addition of H_3PO_3 to extract the dissolved CO_2 into the headspace. Gas samples were drawn from the sample flasks by an airtight syringe (QUINTRON) through one of the sleeve stoppers of the flasks. The headspace gas samples were analyzed by a highly sensitive CO_2 isotope analyzer, called an integrated cavity output spectrometer (ICOS). To study the effect of CO_2 on the isotopic exchange reaction, we injected 1000, 2000, 5000, 10 000, and 50 000 ppm pure CO_2 gases into the five separate flasks. The concentration of CO_2 was measured by a laser-based ICOS spectrometer. Similarly, we studied the influence of temperature, pH, and labeled water ($H_2{}^{18}O$) on the isotopic exchange reaction. All results were compared with the blank. The study received ethical permission from the Institutional Ethics Committee of Vivekananda Institute of Medical Sciences (Registration No. ECR/62/Inst/WB/2013), Kolkata.

RESULTS AND DISCUSSION

In this study, we first monitored the production of $^{12}C^{18}O^{16}O$ isotope due to isotopic exchange between ^{16}O of CO_2 and ^{18}O of H_2O during the in vitro biochemical reaction of $^{12}C^{16}O^{16}O$ and $H_2{}^{18}O$. To investigate the feasibility of this exchange phenomenon to estimate the erythrocyte CA activity, pure carbon dioxide gas (5% CO_2) was injected into the flasks containing hemolysate and ^{18}O-labeled H_2O in closed round-bottom flasks.

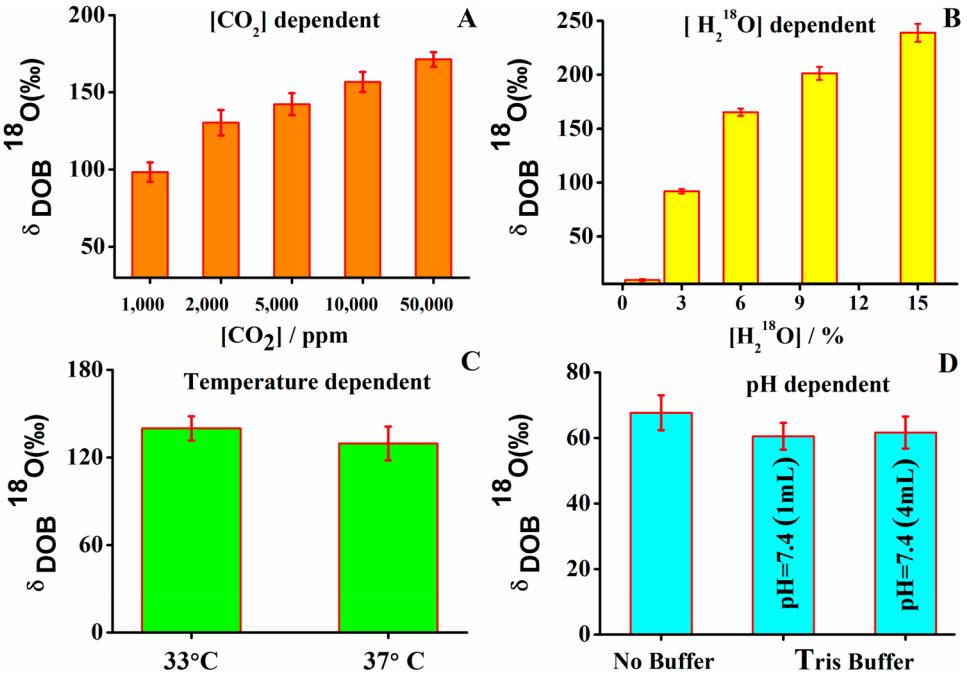


Figure 1. Effects of $[CO_2]$, $[H_2{}^{18}O]$, temperature, and pH on isotopic exchange reaction between ^{16}O-isotope of CO_2 and ^{18}O-isotope of $H_2{}^{18}O$ within the closed flasks. Compositions of flasks are described as (A) $[H_2{}^{18}O]$ = 10‰, temperature = 37 °C, pH = 7.4; (B) $[CO_2]$ = 5%, temperature = 37 °C, pH = 7.4; (C) $[CO_2]$ = 5%, $[H_2{}^{18}O]$ = 5‰, pH = 7.4; (D) $[CO_2]$ = 5%, $[H_2{}^{18}O]$ = 3‰, temperature = 37 °C.

We artificially prepared a wide variety of hemolysate solutions with various CA activities (prepared from blood samples) within the flasks by addition of CA inhibitor (acetazolamide) at desired concentrations. However, it is noteworthy that the kinetics of a chemical reaction primarily depends on several factors such as concentrations of reactants ($[CO_2]$ and $[H_2{}^{18}O]$), temperature, etc.

To investigate the effect of CO_2 on this isotopic exchange reaction, we performed the reaction at a wide variety of CO_2 concentrations ranging from 1000 to 50 000 ppm. Our study demonstrated that the gradual increment of $[CO_2]$ facilitates the rate of the isotopic fractionation reaction to produce ^{18}O-enriched CO_2 (Figure 1A) within the flasks, suggesting the alteration in cellular produced carbon dioxide during the metabolism in human body due to variation of subject-specific basal metabolic rate (BMR) would have a strong influence on exchange kinetics.

Next, to examine the influence of ^{18}O of H_2O, we further investigated the exchange kinetics in the presence of $H_2{}^{18}O$ at multiple concentrations (Figure 1B). The gradual increase of ^{18}O-isotope of CO_2 was found with the increment of $[H_2{}^{18}O]$, suggesting the alteration of individual's $[H_2{}^{18}O]$ in the body can largely alter the rate of the isotopic exchange reaction. In addition, to gain insight into the effect of temperature on this in vitro reaction, we further examined the reaction kinetics with variation of temperatures. Here, we found that the rate of ^{18}O-isotopic production was not affected with a slight increment of temperature (Figure 1C). This observation suggests that the subject-specific variation of body temperature may not alter the kinetics of fractionation reaction during the physiological process in the human body. Now, pH is an important factor to regulate the reaction kinetics. To check the pH dependency on this reaction, we next performed our study at a medium without buffer (pH changes during the progress of the reaction) solution and compared our results with the experiments which were carried out at a particular pH (maintained by tris buffer). Here, the rate of the exchange reaction was found to be altered in buffer medium as compared to nonbuffer medium, indicating that the variation of pH may alter the rate of exchange reaction (Figure 1D).

It is noteworthy that we need to consider all the factors including CO_2, $H_2{}^{18}O$, pH, and temperature during our study to mimic the isotopic reaction in human body.

Therefore, we next performed the whole study with $[CO_2]$ = 50 000 ppm, $[H_2{}^{18}O]$ = 5‰, pH= 7.4, and temperature = 37 °C to create the environment equivalent to that of a human body and subsequently monitored the exchange kinetics at wide varieties of CA activity within the sample flasks (Figure 2). Here, we found that when acetazolamide (CA-inhibitor) inhibited the total CA activity in medium, the ^{18}O-enriched

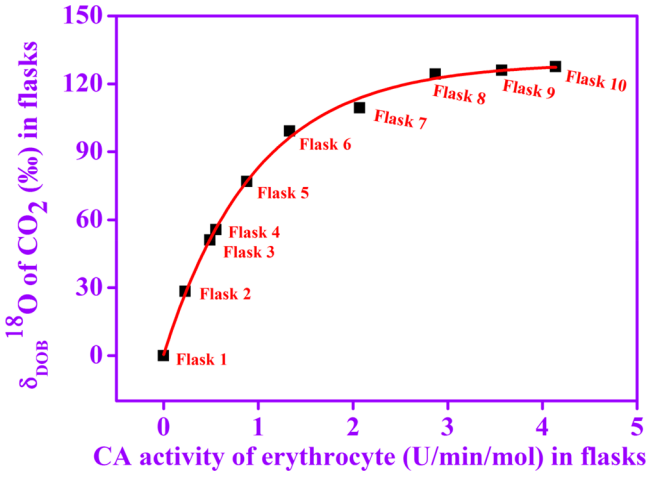


Figure 2. Fittings of the kinetics curve of isotopic exchange reaction within the sample flasks.

CO_2 was found to have almost disappeared in the sample flask. A gradual increment of CA activity in erythrocytes promotes the exchange kinetics to produce more and more ^{18}O-isotope in reaction medium. The kinetics of isotopic exchange reaction was obtained by plotting the ^{18}O-isotope of CO_2 as a function of CA activity. Here, we fitted the curve with the acquired experimental data. We observed that the isotopic exchange reaction followed the first order reaction kinetics. The rate equation can be expressed as follows: $y = A_1 \exp(-x/t) + y_0$, where A_1, y_0, and t are the constants. Here, $A_1 = 128.6$, $y_0 = -129.1$, and $t = 0.97$, where the y represents the ^{18}O-isotope of CO_2 ($\delta_{DOB}{}^{18}O‰$) and x represents the CA activity. This equation describes the production of the oxygen-18 isotope of CO_2 as a function of enzymatic activity of CA. From the knowledge of the ^{18}O-isotope, one can estimate the CA activity quantitatively by utilizing the above equation. This equation shows the feasibility of carbon dioxide isotope analysis by means of ^{18}O-isotopes for estimation of carbonic anhydrase activity. On the basis of a few experiments, our study exhibited a considerable reproducibility of the results and exhibited accurancy ~92%. This assay can be applied for rapid and simple quantification of CA activity in an alternative way instead of a traditional blood-based method.

CONCLUSION

In this study, our findings suggest a new method for quantitative estimation of CA activity from analysis of the oxygen-18 isotope of CO_2. This study is limited to monitor the CA activity above 3 μmol/min/mL as the correlation curve gets saturated in the higher range of CA activity. However, this new method may be applicable to estimate the CA activities within the specified limiting value in various CA-associated disorders. Here, we created the environment similar to that of a human body to monitor the isotopic fractionation reaction which is occurring in a human body. When the conventional method consists of several limitations associated with the tedious and expensive process of sample collection and its preparation to estimate the CA activity, our method shows a new approach to track the CA activity from the measurement of the ^{18}O-isotope of CO_2. One of the advantages of our proposed method is that it may facilitate the rapid screening of carbonic-anhydrase-associated disorders like open-angle glaucoma, mountain sickness, osteoporosis, and neurological disorders, etc., in future days. Moreover, new insights into the linkages between the ^{18}O-isotope of CO_2 and CA activity in a red blood cell will help to treat and prevent the complications caused due to enhancement of carbonic anhydrase enzymatic activity in the human body. However, further research is necessary to validate this method, but the proof-of-concept of this new method has been established through our study.

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Notes

The authors declare no competing financial interest.

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