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*Anal. Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.7b04572 • Publication Date (Web): 15 Dec 2017

# A new strategy for in-vitro determination of carbonic anhydrase activity from analysis of oxygen-18 isotopes of $CO_2$

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Keywords: Carbonic anhydrase, oxygen-18 isotope of $CO_2$, isotopic exchange reaction

Date: 14th December, 2017
**Figures: 2**

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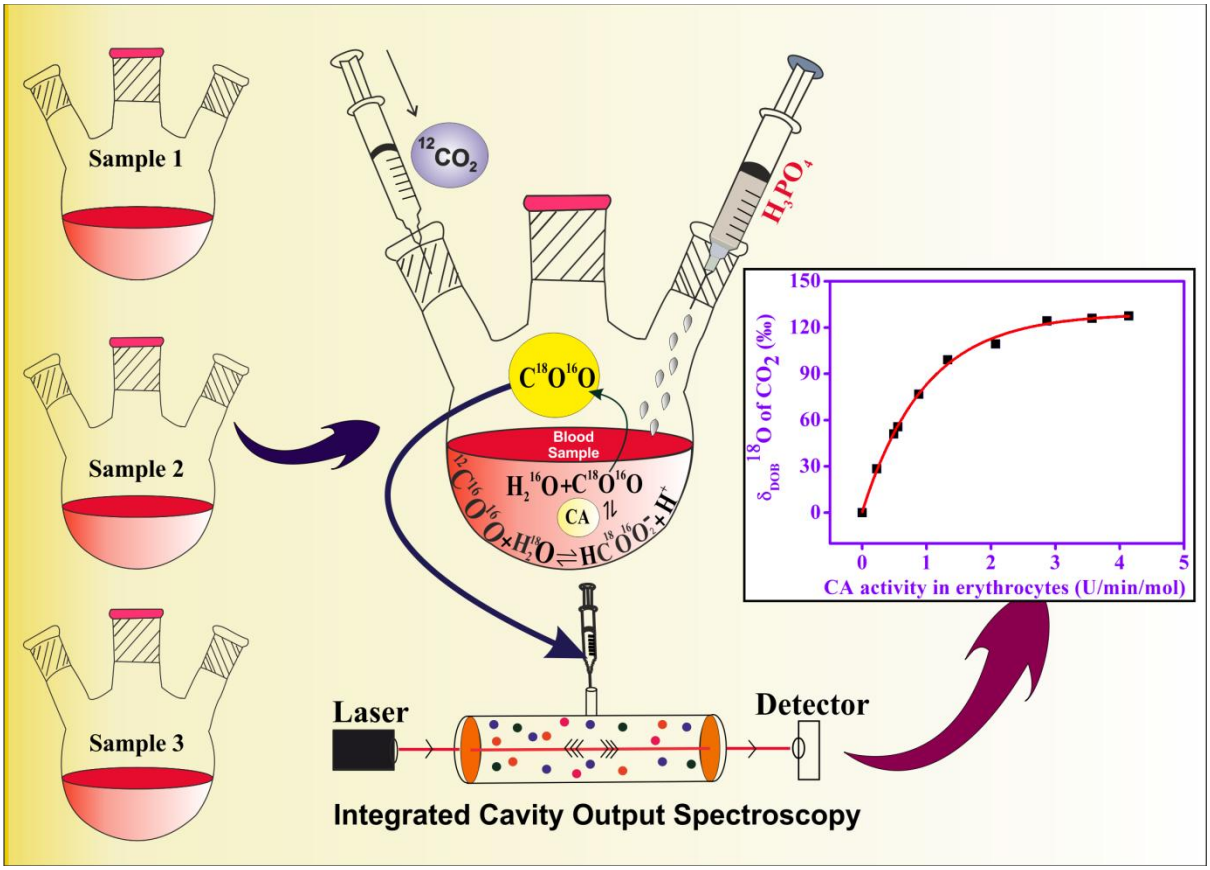
## TOC:




## Abstract:

The oxygen-18 isotopic ($^{18}O$) composition in $CO_2$ provides important insight into the variation of rate in isotopic fractionation reaction regulated by carbonic anydrase (CA) metalloenzyme. This work aims to employ $^{18}O$-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 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 $37^0$C to create the equivalent physiological environment as of human body and monitored the exchange kinetics with variations of CA activities and subsequently we derived the quantitative relation between $^{18}$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.

## 1. Text:

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 human. Sixteen isoforms of α-CAs (CA I to CA VI, CA IX, CA XI, CA XII, CA XIII, CA XIV and CA XV) so far have been isolated 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 body. During the last 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 five to six times higher than CA II, but it shows only 15% of activity as compared to 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 to develop 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 tedious and expensive process including blood sample collection, prolong time for lab processing and subsequently analysis by suitable spectroscopic technique. The assay is based on the spectrophotometric measurement of para-nitro phenol from hydrolysis of para-nitro phenyl acetate in 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 prolong time, suggesting an alternate approach is desperately needed to overcome the above issues.

Early studies suggest[13-16] 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 $^{12}C^{18}O^{16}O$ 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 physiological process has a large impact on isotopic composition of carbon dioxide in human body. The $^{18}O$-isotope may provide useful information for the estimation of erythrocytes 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 non-invasively estimate the CA activity in erythrocytes from

monitoring of oxygen-18 isotopes of $CO_2$. However, no study till 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 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 of subject-specific body temperature, pH, intracellular $CO_2$ and $^{18}O$ of body $H_2O$. All these factors may alter the exchange kinetics of isotopic reaction resulting in variation of $^{12}C^{18}O^{16}O$ isotopic compositions of $CO_2$ in medium.

## 2. Experimental Analysis

### 2.1. 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 r.p.m. for 15 minutes 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 against 4000 r.p.m. for few minutes. The erythrocytes packs were collected and lysed with ice-cold water. The ghost cells from the hemolysate solution were removed after centrifuging it at 10,000 r.p.m for 30 minutes. Carbonic anhydrase activity was determined from the fresh supernatant solution.

## 2.2 Integrated cavity output spectrometer:

A laser-based high-resolution integrated cavity output spectrometer (CCIA 36-EP, Los Gatos research, USA) 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 in somewhere else.[18,19] The present ICOS system consists of two high-finesse optical cavity (~59 cm long) with two high-reflectivity mirrors (R~99.98%) at its 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 $cm^{-1}$ and 4874.448 $cm^{-1}$ in the $(2,0^0,1)\leftarrow(0,0^0,0)$ 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:

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

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

where, $(\frac{^{18}O}{^{16}O})_{standard}$ is the international standard Vienna Pee Dee Belemnite value i.e. 0.0020672.

## 2.3 Carbonic anhydrase activity measurement:

Carbonic anhydrase activity was measured spectro-photometrically 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 comprised of a 1 cm cuvette containing 100 µL hemolysate, 1.86

mL TRIS buffer, and 20 µL PNPA. The absorbance was measured by a UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer) at 348 nm over the period of 3 minutes. 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 absorbance after 3 min, $A_0$ is the absorbance at 0 min, $5\times10^3\ M^{-1}cm^{-1}$ is the molar absorptivity of p-nitrophenol. The activity was normalized to $4.5\times10^9$ cells/mL.

**2.4 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. 5mL hemolysate along with 5‰ $H_2{}^{18}O$ were out into 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 hrs after addition of $CO_2$ gas to attain the equilibrium. After 2 hrs of the reaction, the acidification of the solution was done by addition of $H_3PO_3$ to extract the dissolved $CO_2$ into headspace. Gas samples were drawn from the sample flasks by an airtight syringe (QUINTRON) through one of the sleeve stoppers of flasks. The headspace gas samples were analyzed by a high-sensitive $CO_2$ isotope analyzer, called integrated cavity output spectrometer (ICOS). To study the effect of $CO_2$ on isotopic exchange reaction, we injected 1000 ppm, 2000 ppm, 5000 ppm, 10,000 ppm 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 the ethical permission from the Institutional Ethics Committee of Vivekananda Institute of Medical Sciences (Registration No. ECR/62/Inst/WB/2013), Kolkata.

## 3. Results and Discussions

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 erythrocytes 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.

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 ([$CO_2$] & [$H_2{}^{18}O$]), temperature etc.

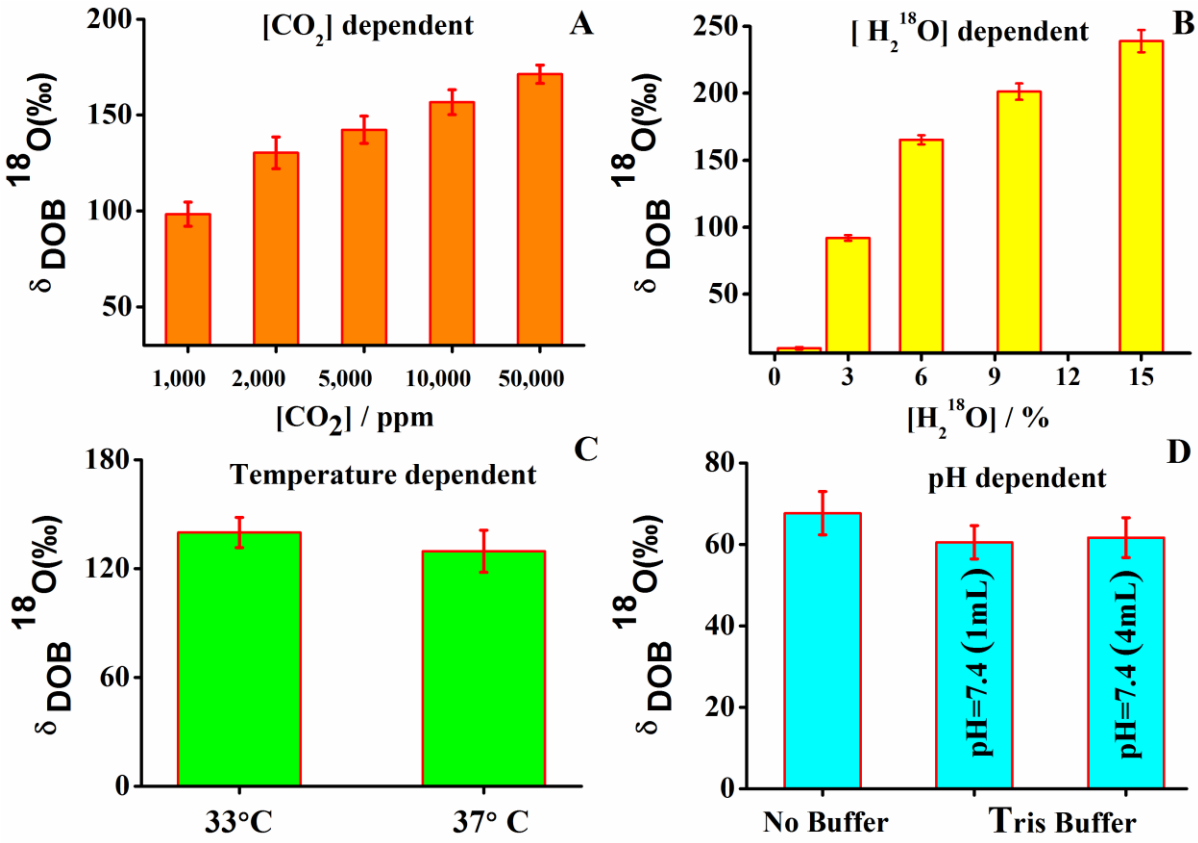



**Figure 1:** *Effects of [$CO_2$], [$H_2{}^{18}O$], temperature and pH on isotopic exchange reaction between $^{16}O$-isotope of $CO_2$ and $^{18}O$-isotope of $H_2{}^{18}O$ within the closed flasks. Compositions of flasks are described as: a) Figure 1A: [$H_2{}^{18}O$] = 10‰, temperature= $37^0$C, pH=7.4; b) Figure 1B: [$CO_2$ ] = 5%, temperature= $37^0$C, pH=7.4; c) Figure 1C: [$CO_2$ ] = 5%, [$H_2{}^{18}O$] =5‰, pH=7.4; d) Figure 1D: [$CO_2$ ] = 5%, [$H_2{}^{18}O$] =3‰, temperature= $37^0$ C*

To investigate the effect of $CO_2$ on this isotopic exchange reaction, we performed the reaction at wide variety of $CO_2$ concentrations ranging from 1000 ppm 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 strong influence on exchange kinetics.

Next to examine the influence of $^{18}O$ of $H_2O$, we further investigated the exchange kinetics in presence of $H_2{}^{18}O$ at multiple concentrations (figure 1B). The gradual increase of $^{18}O$-isotope of $CO_2$ was found with increment of [$H_2{}^{18}O$], suggesting the alteration of individual's [$H_2{}^{18}O$] in body can largely alter the rate of the isotopic exchange reaction. Further 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 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 physiological process in 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 non-buffer medium, indicating 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.

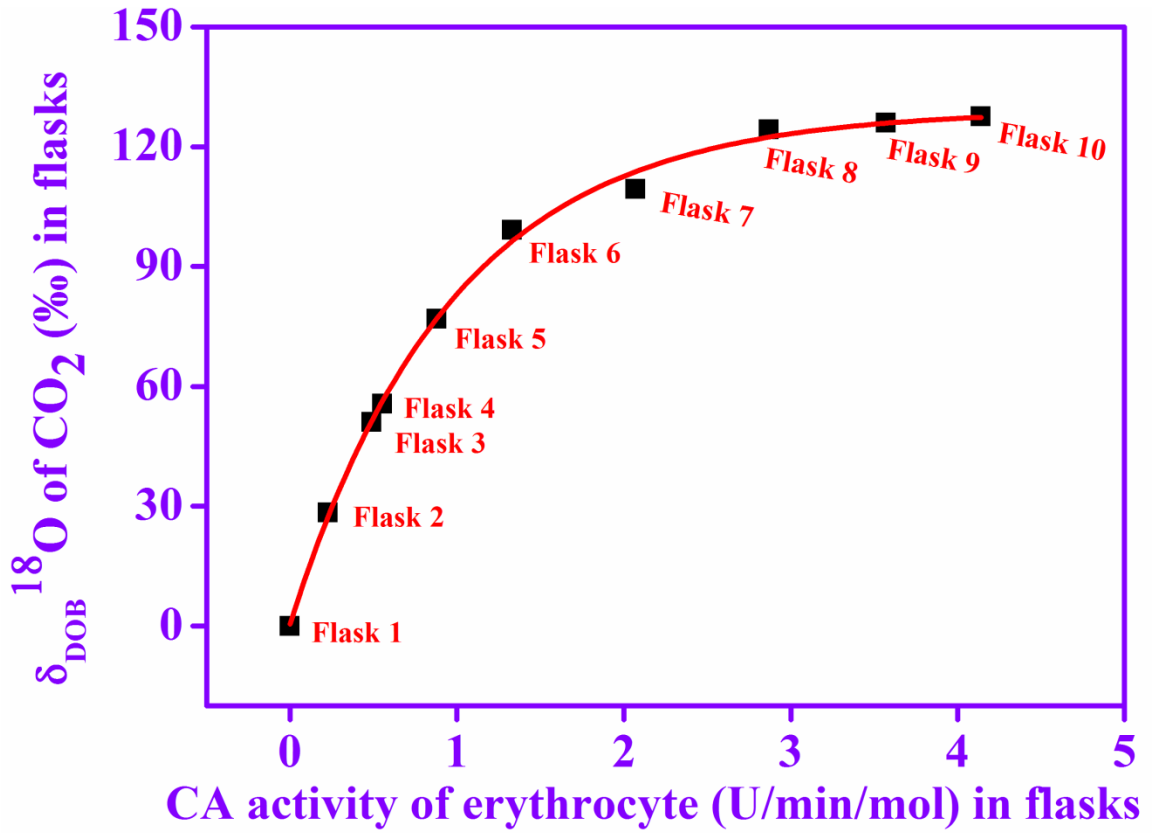



**Figure 2**: *Fittings of the kinetics curve of isotopic exchange reaction within the sample flasks*

Therefore, we next performed the whole study with [$CO_2$] = 50,000 ppm, [$H_2{}^{18}O$] = 5‰, pH= 7.4 and temperature =37$^0$C to create the equivalent environment as of human body and subsequently monitored the exchange kinetics at a 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 $CO_2$ was found to be almost disappeared in the sample flask. 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 A1, y0 and t are the constants. Here, $A_1$= 128.6, $y_0$ = -129.1 and t=0.97, whereas 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 oxygen-18 isotope of $CO_2$ as a function of enzymatic activity of CA. From the knowledge of $^{18}O$-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. Based on few experiments, our study exhibited a considerable reproducibility of the results and exhibited the accurancy ~92%. This assay can be applied for rapid and simple quantification of CA activity in alternative way instead of traditional blood-based method.

## 4. Conclusion:

In this study, our findings suggest a new method for quantitative estimation of CA activity from analysis of 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 at 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 similar environment as of human body to monitor the isotopic fractionation reaction which is occurring in human body. When the conventional method consists of several limitations associated with 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 $^{18}O$-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 $^{18}O$-isotope of $CO_2$ and CA activity in red blood cell will help to treat and prevent the complications caused due to enhancement of carbonic anhydrase enzymatic activity in 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.

## 5. Acknowledgements

M. Pradhan acknowledges the funding from the Technical Research Centre (TRC) (No. All1/64/SNB/2014(C)) of Satyendra Nath Bose National Centre for Basic Sciences, India. S. Mandal and C. Ghosh thank to S. N. Bose Centre for PhD fellowships, whereas M. Pal acknowledges to the Department of Science & Technology (DST, India) for Inspire Fellowships.

## 6. References

1. Henry, R.P. *Annu. Rev. Physiol.* **1996**, *58***,** 523-538.
2. Kondo, T. *et al. Clinica Chimica Acta* **1987**, *116*, 227-236.
3. Supuran, C.T. *Nature Reviews Drug Discovery* **2008**,*7*,168-181
4. Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Pat*. **2006**, *16*, 1627–1664.
5. Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev*. **2003**, *23*, 146–189.
6. Kondo, T. *et al. Clinica Chimica Acta* **1987**, *116*, 227-236.
7. Briganti, F. *et al. Biochemistry* **1997**, *36*, 10384–10392.
8. Thiry, A. *et al. Trends Pharmacol. Sci*. **2006**, *27*, 566–573.
9. Pastorekova, S. *et al. J. Enzyme Inhib. Med. Chem*. **2004**, *19*, 199–229.
10. Nishimori, I. *et al. Bioorg. Med. Chem*. **2007**, **1***5*, 7229–7236.
11. Parui, R.; Gambhir, K. K.; Mehrotra, P. P. *Biochem. Int*. **1991**, *23***,** 779-789.

12. Gambhir, K. K.; Oates, P.; Verma, M.; Temam, S; Cheatham, W. *Ann. N. Y. Acad. Sci.* **1997**, *827*, 163-169.

13. Enns, T. *Science* **1967**, *155*, 44-47.

14. Epstein, S.; Zeiri, L. *Proc. Natl. Acad. Sci. USA*. **1988**, *85***,** 1727-1731.

15. Forster, R. E.; Gros, G., Lin, L.; Ono, Y.; Wunder, M. *Proc. Natl. Acad. Sci. USA*. **1998**, *95*, 15815–15820.

16. Gillon, J.; Yakir, D. *Science* **2001**, *291*, 2584–2587.

17. Gambhir, K. K.; Ornasir, J.; Headings, V.; Bonar, A. *Biochemical Genetics* **2007**, *45*, 431-439.

18. Crosson, E. R. *et al. Anal. Chem*. **2002**, *74*, 2003-2007.

19. Barker, S. L. L.; Dipple, G. M.; Dong, F.; Baer, D. S. *Anal. Chem*. **2011**, *83***,** 2220-2226.

20. Armstrong, J. McD.; Myers, D. V.; Verpoorte, J. A.; Edsall, J. T. *J. Biol. Chem*. **1966**, *241*, 5137-5149.

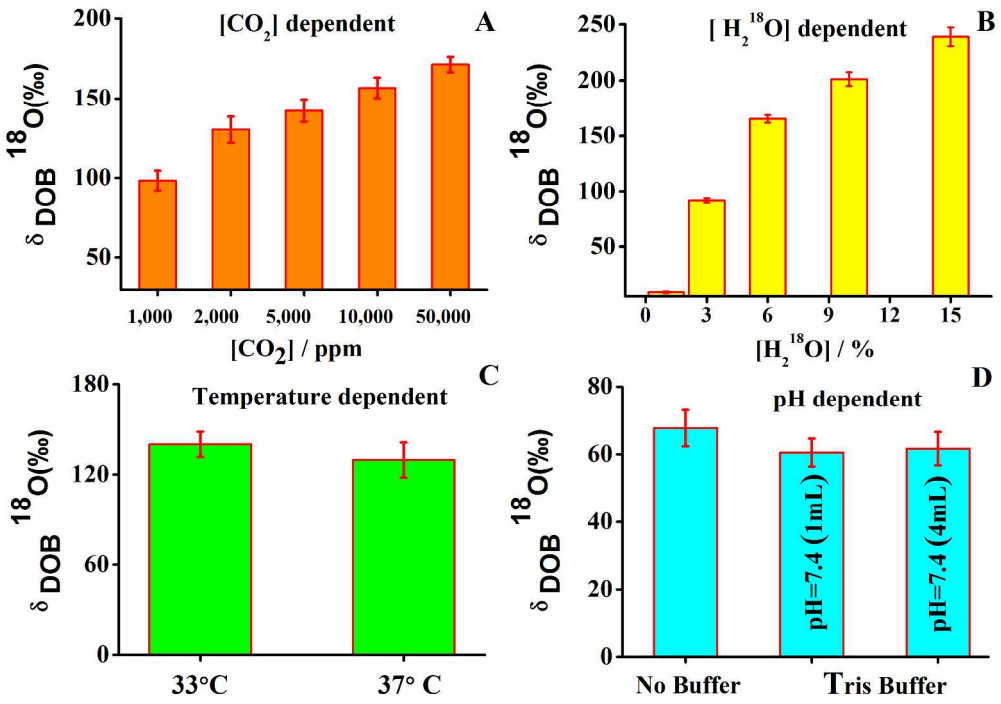



Figure 1 Effects of $[CO_2]$, $[H_2{}^{18}O]$, temperature and pH on isotopic exchange reaction between $^{16}O$-isotope of $CO_2$ and $^{18}O$-isotope of $H_2{}^{18}O$ within the closed flasks. Compositions of flasks are described as: a) Figure 1A: $[H_2{}^{18}O]$ = 10‰, temperature= $37^0$C, pH=7.4; b) Figure 1B: $[CO_2]$ = 5%, temperature= $37^0$C, pH=7.4; c) Figure 1C: $[CO_2]$ = 5%, $[H_2{}^{18}O]$ =5‰, pH=7.4; d) Figure 1D: $[CO_2]$ = 5%, $[H2{}^{18}O]$ =3‰, temperature= $37^0$ C

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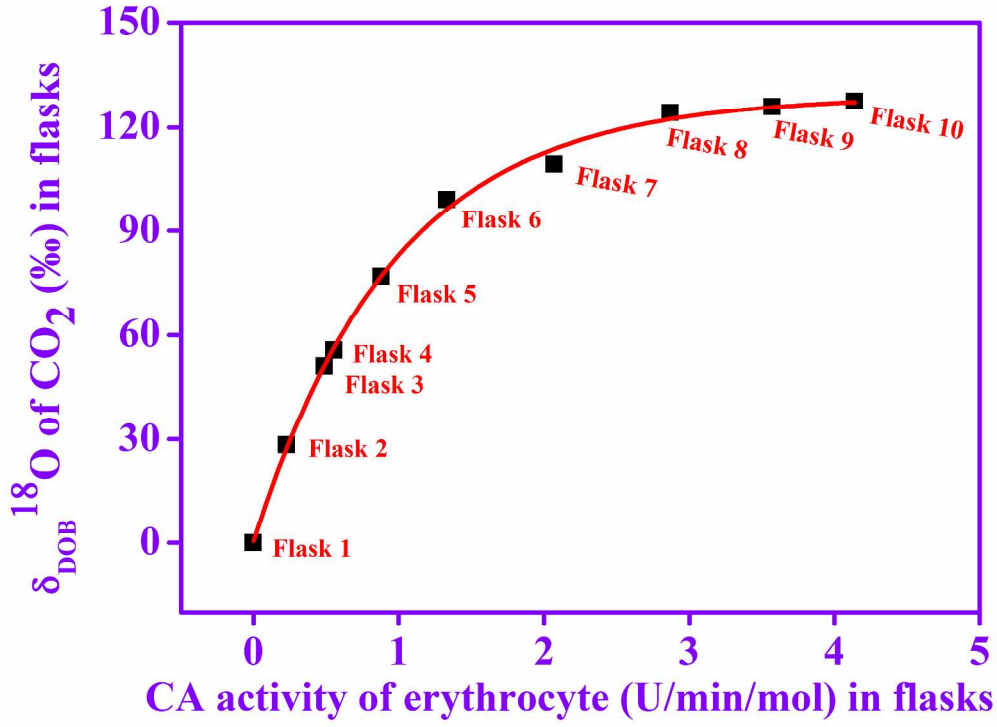


Figure 2 Fittings of the kinetics curve of isotopic exchange reaction within the sample flasks

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