Junghans, P., **Strauch, G.**, Voigt, J. (2020):
In vitro application of carbonic anhydrase to accelerate the equilibration of ^{18}O between H_2O and CO_2 for the rapid measurement of $^{18}O/^{16}O$ isotope ratios in aqueous samples
Isot. Environ. Health Stud. **56** (3), 314 – 323

In vitro application of carbonic anhydrase to accelerate the equilibration of ^{18}O between H_2O and CO_2 for the rapid measurement of $^{18}O/^{16}O$ isotope ratios in aqueous samples

Peter Junghans*[a], Gerhard Strauch[b] and Jürgen Voigt[a]

[a]*Institute of Nutritional Physiology "Oskar Kellner", Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany,* [b]*Helmholtz-Zentrum für Umweltforschung – UFZ, Leipzig, Germany*

**Correspondence to*: Peter Junghans Email: peter_junghans@web.de

Abstract

A novel method of the accelerated equilibration of ^{18}O between CO_2 and H_2O for the measurement of the $^{18}O/^{16}O$ isotope ratios in aqueous samples with natural isotope abundances is presented. This rapid equilibrium method is based on the *in vitro* application of the enzyme carbonic anhydrase (CA). The CA from bovine erythrocytes was adsorptively fixed to 3-mm glass beads with an etched surface. After addition of this carrier-fixed CA catalyst to the water sample, the isotope equilibrium was already reached after 1 h. The previously used non-catalyzed ^{18}O isotope exchange in water samples needs about 24 h. Whole blood samples also showed fast ^{18}O isotope equilibration, which definitely results from native presence of CA in erythrocytes. By shortening the time for sample preparation, the CA catalyzed technique can significantly increase the throughput of the samples to be measured, and also ^{18}O and ^{2}H measurement by means of isotope ratio mass spectrometry (IRMS) may be synchronized. The ^{2}H and ^{18}O sample preparation can be performed in the same reaction vessel because cross-effects at the simultaneous use of Pt and CA catalysts do not occur.

Keywords: blood; carbonic anhydrase; hydrogen-2; isotope equilibrium; isotope ratio mass spectrometry; oxygen-18; plasma; rapid isotope analysis; water

1. Introduction

The stable isotope ^{18}O plays an important role for studying dynamic processes in different fields such as hydrogeology, paleoclimatology [1], food control (origin, authenticity of products) [2,3], forensic science [4], physiology and medicine [5,6]. One dominant application of ^{18}O and ^{2}H in medicine and nutritional physiology is the investigation of energy metabolism using the doubly labelled water (DLW) technique [7–12].

To determine ^{18}O and ^{2}H abundances in water and biological aqueous samples such as urine, blood plasma, saliva etc., the isotope equilibrium procedure is widely used [13–17]. This procedure is based on the transfer of ^{18}O and ^{2}H from the H_2O of the aqueous sample to CO_2 and H_2 which are suitable gases for measurement by isotope ratio mass spectrometry (IRMS). The prior removal of constituents from the aqueous sample such as proteins, amino acids, urea, inorganic constituents etc. is actually not required. However, some authors isolated the water from biological fluids (plasma and urine) by cryodistillation before the equilibration technique was applied [14].

A more direct and rapid system for the analysis of $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of water is the thermal conversion elemental analyzer (TC/EA) coupled with IRMS, which converts water into H_2 and CO at 1420 °C [18,19]. The previous isolation of water from the aqueous sample, e.g. blood plasma is required to avoid isotope dilution by constituents such as amino acids, proteins etc. This system was used to measure $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios in plasma of rats [20].

An alternative approach to IRMS for water isotope analyses is laser absorption spectroscopy. This technique is less expensive than IRMS and allows less tedious sample preparation. It is described to measure ^{18}O and ^{2}H in urine of humans [21]. Further discussion on this technique is beyond the scope of this paper.

Considering the respective exchange of ^{18}O or ^{2}H between H_2O of the aqueous sample and CO_2 or H_2 until isotope equilibrium, Cohn and Urey [22] performed pioneering studies on this ^{18}O exchange reaction already in 1938. The non-catalyzed ^{18}O exchange $H_2{}^{18}O + CO_2 \leftrightarrow H_2{}^{16}O + C^{18}O^{16}O$ until the isotope equilibrium lasts in dependence on the experimental conditions about 12 to 72 h [14,23]. Contrary, the isotope equilibrium $^{2}H^{1}HO + H_2 \leftrightarrow H_2O + {}^{2}H^{1}H$ is reached rapidly by means of a platinum catalyst after about 2 h [24]. The slow ^{18}O isotope exchange impedes a rapid

analysis of ^{18}O and, thus, the synchronization of both ^{2}H and ^{18}O analyses. Therefore, the acceleration of the ^{18}O isotope exchange would be desirable to solve this problem.

In humans and most animals the exchange between oxygen in H_2O and CO_2 is efficiently accelerated by the enzyme carbonic anhydrase (CA). CA represents a family of metalloenzymes, mostly with zinc as active site. CA occurs in different species, e.g. vertebrates and mammals. The major physiological role of CA is the maintenance of essential life functions such as pH homeostasis. Also, respiration is only possible by the presence of CA, i.e. the fixation of CO_2 in bicarbonate or release of CO_2 from its fixed chemical form in bicarbonate by exhalation via the lungs [25].

The aim of this study is to examine if and to what extent the isotope exchange between the oxygen isotope ^{18}O in water and the reactant gas CO_2 may be accelerated by *in vitro* application of carrier-fixed CA and thus enables a rapid measurement of the $^{18}O/^{16}O$ isotope ratios in water or biological fluids. For this purpose a suitable CA preparation was tested. A further objective of this paper is to investigate the combined preparation of aqueous samples by means of Pt and CA catalysts for the accelerated ^{2}H and ^{18}O isotope exchange in the same reaction vessel.

2. Materials and methods

2.1. Water and blood samples

In this study, water, whole blood and its plasma with natural isotope abundances were investigated in three experiments. Water samples were used from the working standard of the mass spectrometry laboratory of the Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany (Dummerstorf standard water, DSW). Blood was obtained from two bulls of a F2 German Holstein x Charolais cross (body mass: about 330 kg; age: 4.5 months) [26]. The experimental protocol was approved by the Ethics Committee of the Ministry of Nutrition, Agriculture, Forestry, and Fishery, Schwerin, State Mecklenburg–Vorpommern, Germany (LVL M-V/310-4/7221.3–2.1–015/02).

Blood samples were collected in Li-heparinate monovettes (Sarstedt, Nürnberg, Germany). Monovettes were gently shaken to obtain whole blood or were centrifuged at 1450 × g for 15 min at 4 °C to obtain blood plasma. Each whole blood and blood plasma sample was pooled to obtain homogenous sample material and then stored at -20 °C until use.

To study the effect of CA on the rate of the ^{18}O isotope exchange between water (from DSW or blood plasma) and CO_2 under different conditions the CA was fixed on glass beads, described below, and added to the samples (experiments 1 and 2). In a further study (experiment 3) CA and Pt catalysts (as platinum rods, Thermo Scientific, Bremen) were placed in the same exetainers with DSW samples to investigate possible cross-effects of the catalysts on the ^{18}O and ^{2}H isotope exchange processes between H_2O and CO_2 as well as H_2O and H_2, respectively. Experiments 1 to 3 are summarized in Table 1.

2.2. Preparation of the CA glass beads

The CA from bovine erythrocytes (as a lyophilized powder, >2000 U/mg, Fluka Chemie GmbH, Buchs, Switzerland, no. 21803) was fixed by adsorption to glass beads with etched surface (diameter: 3 mm). Briefly, 2 mg CA were dissolved in 10 ml distilled water. Thereafter, about 200 glass beads were given to the CA solution and shaken for 30 minutes. Then, the glass beads coated with CA were given to a glass frit and dried by means of an N_2 stream at room temperature and finally stored at –20 °C until use.

2.3. Isotope analysis by mass spectrometry

The $^{18}O/^{16}O$ and $^{2}H/^{1}H$ isotope ratios of water, whole blood and plasma samples with natural ^{18}O and ^{2}H abundances were measured by means of an isotope ratio mass spectrometer (DELTA plus XL, Thermoquest, Bremen, Germany) coupled with a Gas Bench II (Finnigan, Bremen, Germany) [12]. Equilibration methods of ^{18}O and ^{2}H with automatized systems are extensively described in the literature [13,15,16,24,27]. All samples were measured as 5 replicates. The averaged isotope values were used for subsequent calculations. The precision of the measurement amounted to $\delta^{18}O = \pm 0.2$ ‰ and $\delta^{2}H = \pm 1.3$ ‰.

2.4. Experiment 1 – water samples

500 µL water (DSW) and one glass bead with CA, prepared as described above, were given into a 13-ml Exetainer® (Labco Ltd., Buckinghamshire, UK) with round bottom. Parallel, 500 µL water (DSW) and one glass bead *without* CA were placed into an exetainer. The exetainers were sealed with a rubber septum and flushed with reactant

gas (0.3 % CO_2 in helium) for 5 min at 100 mL/min. After equilibration times of 1, 3, 6, and 24 h at 25 °C, the $^{18}O/^{16}O$ isotope ratio of CO_2 in the headspace was measured by IRMS.

The $^{18}O/^{16}O$ isotope ratio of CO_2 was additionally measured in DSW samples at 1, 3, 6 and 24 h under the following conditions: (a) samples + freshly prepared CA glass beads, (b) samples + CA glass beads defrosted from –20 °C after storage over 14 days (= CA-20), (c) samples + CA-20 treated with ultrasound for 5 min, and (d) samples + CA-20 heated in a water bath at 50 °C for 15 min. In the following experiments 2 and 3 only defrosted CA glass beads, as described in (b), were used. Therefore, the term CA-20 was simplified to CA.

2.5. Experiment 2 – blood samples

500 µL of blood plasma and one glass bead with CA were given in an exetainer. Furthermore, 500 µl whole blood (fresh or defrosted from –20 °C) were placed into exetainers *without* CA addition. The following steps were carried out as described in experiment 1.

2.6. Experiment 3 – Combined measurement of ^{18}O and ^{2}H in water samples

For the combined ^{18}O and ^{2}H measurement DSW samples (as described in experiment 1) were placed together with the CA and Pt catalysts in the same exetainer. The following combinations were examined: DSW alone, DSW+CA, DSW+Pt and DSW+CA+Pt.

2.7. Calculations

The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios were measured relative to corresponding laboratory working standards, converted to $\delta^{2}H$ and $\delta^{18}O$ values (expressed in ‰) and, thereafter, normalized against the international water standards Vienna Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) as described in the literature [28,29].

2.8. Statistical analyses

Results are presented as mean values with standard deviations. Differences among

means of the experimental variants used were calculated by the Student's *t*-test or the Welsh test [30]. Statistical significance was accepted if the null hypothesis was rejected with $P < 0.05$.

3. Results

3.1. Water samples

The CA fixed on glass beads applied to water (DSW) samples accelerates the time of equilibration of the ^{18}O exchange between water and CO_2. The isotope equilibrium is already completed after 1 h as shown in Figure 1. The isotope exchange needs about 24 h in water samples without CA, where after 1 h the $\delta^{18}O$ value amounts to about -6 ‰, which is clearly far from the final equilibrium value at 24 h.

The catalytic effect by CA was not changed if the CA glass beads were stored at –20 °C for 2 weeks before their application as compared to freshly prepared CA glass beads (Figure 2). Also, the treatment of water samples + CA with ultrasound for 5 min did not affect the time until the achievement of the isotope equilibrium. When the water samples + CA were heated at 50 °C in the water bath for 15 min the $\delta^{18}O$ values were increased as compared to the other treatments (overall mean±SD (1, 3, 6 and 24 h): 0.39±0.12 (heated) vs. 0.02±0.09 (CA fresh, defrosted, ultra sound), $p<0.001$).

3.2. Samples of blood plasma and whole blood

In blood plasma the exchange of ^{18}O between water and CO_2 is considerably different from 'pure' water samples as shown in Figure 3. Thus, the $\delta^{18}O$ value at 1 h of equilibration time has reached about 27 % of the final value at 24 h. The CA added to plasma significantly speeds up the isotope exchange. In samples of whole blood (fresh or defrosted from –20 °C) the isotope equilibrium is already reached after 1 h without added CA. The $\delta^{18}O$ value of defrosted blood is about 12 % higher at 24 h as compared to fresh blood. The $\delta^{18}O$ values of DSW samples without and with CA addition are presented in Figure 3 for the direct comparison to plasma and blood. The $\delta^{18}O$ values of plasma and blood are about 3-4 ‰ (δ units) higher than those of DSW.

3.3. Combined measurement of ^{18}O and ^{2}H

For the ^{18}O and ^{2}H measurement the simultaneous sample preparation of DSW samples using Pt and CA catalysts in the same exetainer provides results which are shown in Figure 4. The Pt catalyst did not influence the ^{18}O exchange. The ^{2}H results were not significantly affected by the CA.

4. Discussion

The isotope exchange $H_2{}^{18}O + C^{16}O_2 \leftrightarrow H_2{}^{16}O + C^{18}O^{16}O$ of water samples needs about 12 to 72 h in conventional procedures until the isotope equilibrium is reached [14,15,23,31]. The equilibration process can be speeded up to some extent by shaking and minimizing of the reaction vessel to reduce the head space [14,32]. In contrast, the isotope exchange $^{2}HO^{1}H + {}^{1}H_2 \leftrightarrow H_2O + {}^{2}H^{1}H$ lasts only 2 to 3 h by using a Pt catalyst [24].

In this paper it has been shown, to our knowledge for the first time, that with the *in vitro* application of CA, fixed on glass beads, the isotope exchange between $H_2{}^{18}O$ and $C^{16}O_2$ of water samples (DSW) can considerably decrease the time until the isotope equilibrium is reached, i.e. from >12 h to 1–2 h (Figures 1 and 3). Thus, the ^{18}O equilibration procedure by using CA can be synchronized with the Pt-catalyzed ^{2}H equilibrium technique in aqueous samples. The accelerated exchange reaction of ^{18}O between CO_2 and water based on the catalytic *in vitro* effect of CA was already studied by Meldrum and Roughton [33]; however, later on, this option has been only rarely applied for the mass spectrometric ^{18}O isotope analysis using the equilibrium technique [5]. However, vice versa, the ^{18}O exchange kinetics between water and CO_2 was applied for the *in vitro* determination of CA activity, which can diagnose CA-associated disorders in humans [34].

To examine several conditions of the sample preparation on the ^{18}O isotope exchange, water samples with freshly prepared CA glass beads, with CA after its storage at –20 °C, water samples + CA treated by ultrasound and heat were investigated. The samples treated with freshly prepared CA glass beads showed no different $\delta^{18}O$ values against those which were stored for 2 weeks and then defrosted. Also, water samples + CA treated by ultrasound did not show different $\delta^{18}O$ values as compared to the latter ones. Heat affected the ^{18}O isotope exchange by a slight increase of the $\delta^{18}O$

value against the other treatments at 1, 3 and 6 h (Figure 2). After 24 h the 'heat' effect disappeared. The plausible explanation could be that heat speeds up the reaction rates of the isotope exchange, whereas the transfer of ^{18}O from water to CO_2 gas is increased. However, after the time course of 24 h the system returns to the equilibrium. Heat-related alterations of the enzymatic activity of CA are not expected because the CA was found to be catalytically intact below 60 °C [35]. Nevertheless, the 'heat' effect needs further investigations for clarifying the physical process.

The application of the equilibration procedure on ^{18}O was also studied in blood plasma and whole. The ^{18}O isotope exchange between water and CO_2 will not take place only in 'pure' water, but also in aqueous samples with inorganic and organic constituents. Therefore, it was suggested that plasma without CA addition will show a behavior similar to that of water. The results only partly confirm our suggestion (Figure 3). The isotope equilibrium was already reached after 3 h. Normally, blood plasma should not contain CA because it is localized in the erythrocytes [25,36]. Therefore, it must be assumed that small amounts of CA were transferred during the centrifugation of heparinized blood from the erythrocytes to plasma, i.e. uncontrolled hemolysis took place. This explanation is supported by the following results. It was found immediate isotope equilibrium using fresh whole blood, and still more in blood defrosted from –20 °C. In the latter case the CA localized in the erythrocytes will be released by the freezing/defrosting process as expected (Figure 3). For studies of energy expenditure using the DLW measurements of ^{18}O and ^{2}H abundances in biological body fluids such as blood plasma, urine or saliva is mostly used. Conventionally, after cryodistillation of plasma, urine or saliva the resulting 'pure' water samples are prepared separately using the equilibrium techniques, i.e. one aliquot of water sample for ^{18}O and another one for ^{2}H measurements. The reason for this procedure are the different time scales of the equilibration process for ^{18}O (>12 h) and ^{2}H (about 2 h) [24].

As mentioned above, in this paper it could be shown that by use of the CA catalyst the ^{18}O isotope exchange is already completed after 1 to 3 h instead of more than 12 h without CA (Figure 1). Therefore, for the ^{18}O and ^{2}H measurement it was tested the simultaneous sample preparation of water using Pt and CA catalysts in the same exetainer. The results prove that cross-effects did not occur (Figure 4). That means, the Pt catalyst did not influence the ^{18}O exchange and the ^{2}H results were not affected by the CA.

An essential advantage of ‘dry’ glass beads as carrier of CA is that the fixed enzyme amounts are well defined and the entry of extraneous water, e.g. using aqueous CA solutions, is prevented, thus no isotope dilution correction of the measured $^{18}O/^{16}O$ isotope ratio is required. To avoid isotope memory effects, the CA glass beads are only recommended for single use. This is acceptable because CA glass beads are cheap (100 glass beads cost less than 1 €) and easy to prepare.

The presented investigations in water, blood and plasma should be verified in other specimens such as urine, saliva etc. at different sample sizes in future studies. Moreover, ^{18}O and ^{2}H isotopically enriched aqueous samples should be examined.

5. Conclusions

The presented novel *in vitro* CA catalyzed equilibrium technique for ^{18}O measurement provides the isotope results faster than the conventional non-catalyzed technique. By shortening the time for sample preparation to about 2 h, the CA catalyzed technique can significantly increase the throughput of the samples to be measured, and also ^{18}O and ^{2}H IRMS measurements may be synchronized. Both can be important for urgent result requirements, for example in the forensics and emergency medicine or process control of the food industry. The present study also demonstrates that the ^{2}H and ^{18}O sample preparation can be performed in the same reaction vessel because cross-effects at the simultaneous use of Pt and CA catalysts do not occur. The CA fixed on glass beads can be easily prepared and stored for several weeks at –20 °C without loss of enzymatic activity.

Acknowledgements

We thank Ingrid Brüning, Ute Lüdtke, Kirsten Karpati, Axel Fischer and Helmut Scholze for analytical and statistical assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] van Geldern R, Baier A, Subert HL, et al. Pleistocene paleo-groundwater as a pristine fresh water resource in southern Germany – Evidence from stable and radiogenic isotopes. Sci Total Environ. 2014;496:107–115.

[2] Boner M, Förstel H. Stable isotope variation as a tool to trace the authenticity of beef. Anal Bioanal Chem. 2004;378:301–310.

[3] Orellana S, Johansen AM, Gazis C. Geographic classification of U.S. Washington State wines using elemental and water isotope composition. Food Chem X. 2019;1:100007.

[4] Meier-Augenstein W, Fraser I. Forensic isotope analysis leads to identification of a mutilated murder victim. Sci Justice. 2008;48:153–159.

[5] Lifson N, Gordon GB, Visscher MB, et al. The fate of utilized molecular oxygen and the source of the oxygen of respiratory carbon dioxide, studied with the aid of heavy oxygen. J Biol Chem. 1949;180:803–811.

[6] O'Grady SP, Wende AR, Remien CH, et al. Aberrant water homeostasis detected by stable isotope analysis. PLoS ONE. 2010;5:e11699.

[7] Lifson N, McClintock R. Theory of use of the turnover rates of body water for measuring energy and material balance. J Theor Biol. 1966;12:46–74.

[8] Schoeller DA, Ravussin E, Schutz Y, et al. Energy expenditure by doubly labeled water: validation in humans and proposed calculation. Amer J Physiol. 1986;250:R823–830.

[9] Speakman JR. Doubly labelled water: Theory and practice. London: Chapman & Hall; 1997.

[10] Westerterp KR. Doubly labelled water assessment of energy expenditure: principle, practice, and promise. Eur J Appl Physiol. 2017;117:1277–1285.

[11] Junghans P, Derno M, Gehre M, et al. Calorimetric validation of ^{13}C bicarbonate and doubly labelled water method for determining the energy expenditure in goats. Z Ernährungswiss. 1997;36:268–272.

[12] Junghans P, Görs S, Langhammer M, et al. Breath water-based doubly labelled water method for the noninvasive determination of CO_2 production and energy expenditure in mice. Isot Environ Health Stud. 2018;54:561–572.

[13] Brenninkmeijer CA, Morrison PD. An automated system for isotopic equilibration of CO_2 and H_2O for ^{18}O analysis of water. Chem Geol. 1987;66:21–26.

[14] Wong WW, Lee LS, Klein PD. Deuterium and oxygen-18 measurements on microliter samples of urine, plasma, saliva, and human milk. Am J Clin Nutr. 1987;45:905–913.

[15] Schoeller DA, Luke AH. Rapid ^{18}O analysis of CO_2 samples by continuous-flow isotope ratio mass spectrometry. J Mass Spectrom. 1997;32:1332–1336.

[16] De Groot PA, editor. Handbook of stable isotope analytical techniques. Vol. 2. Amsterdam: Elsevier; 2008.

[17] Schierbeek H, Rieken R, Dorst KY, et al. Validation of deuterium and oxygen18 in urine and saliva samples from children using on-line continuous-flow isotope ratio mass spectrometry. Rapid Commun Mass Spectrom. 2009;23:3549–3554.

[18] Gehre M, Geilmann H, Richter J, et al. Continuous flow $^{2}H/^{1}H$ and $^{18}O/^{16}O$ analysis of water samples with dual inlet precision. Rapid Commun Mass Spectrom. 2004;18:2650–2660.

[19] Ripoche N, Ferchaud-Roucher V, Krempf M, et al. D and ^{18}O enrichment measurements in biological fluids in a continuous-flow elemental analyser with an isotope-ratio mass spectrometer using two configurations. J Mass Spectrom. 2006;41:1212–1218.

[20] Richelle M, Darimont C, Piguet-Welsch C, et al. High-throughput simultaneous determination of plasma water deuterium and 18-oxygen enrichment using a high-temperature conversion elemental analyzer with isotope ratio mass spectrometry. Rapid Commun Mass Spectrom. 2004;18:795–798.

[21] Melanson EL, Swibas T, Kohrt WM, et al. Validation of the doubly labeled water method using off-axis integrated cavity output spectroscopy and isotope ratio mass spectrometry. Amer J Physiol. 2018;314:E124–E130.

[22] Cohn M, Urey HC. Oxygen exchange reactions of organic compounds and water. J Amer Chem Soc. 1938;60:679–687.

[23] McMillan DC, Preston T, Taggart DP. Analysis of ^{18}O enrichment in biological fluids by continuous flow-isotope ratio mass spectrometry. Biomed Environ Mass Spectrom. 1989;18:543–546.

[24] Wong WW, Clarke LL. A hydrogen gas–water equilibration method produces accurate and precise stable hydrogen isotope ratio measurements in nutrition studies. J Nutr. 2012;142:2057–2062.

[25] Geers C, Gros G. Carbon dioxide transport and carbonic anhydrase in blood and muscle. Physiol Rev. 2000;80:681–715.

[26] Junghans P, Voigt J, Jentsch W, et al. The ^{13}C bicarbonate dilution technique to determine energy expenditure in young bulls validated by indirect calorimetry. Livest Sci. 2007;110:280–287.

[27] Thielecke F, Brand W, Noack R. Hydrogen isotope determination for small size water samples using an equilibration technique. J Mass Spectrom. 1998;33:342–345.

[28] Prentice AM, editor. International Dietary Energy Consultancy Group. The doubly-labelled water method for measuring energy expenditure: Technical recommendations for use in humans. Cambridge: IDECG/ Vienna: IAEA; 1990.

[29] Nelson ST. A simple, practical methodology for routine VSMOW/SLAP normalization of water samples analyzed by continuous flow methods. Rapid Commun Mass Spectrom. 2000;14:1044–1046.

[30] SAS Institute Inc., SAS/STAT user's guide, version 8. Cary, NC: SAS Institute Inc.; 1999.

[31] Fessenden J, Cook C, Lott M, et al. Rapid ^{18}O analysis of small water and CO_2 samples using a continuous-flow isotope ratio mass spectrometer. Rapid Comm Mass Spectrom. 2002;16:1257–1260.

[32] Roether W. Water–CO_2 exchange set-up for the routine oxygen-18 assay of natural waters. Int J Appl Radiat Isot. 1970;21:379–387.

[33] Meldrum NU, Roughton FJ. The state of carbon dioxide in blood. J Physiol. 1933;80:143–170.

[34] Ghosh C, Mandal S, Pal M, et al. New strategy for in vitro determination of carbonic anhydrase activity from analysis of oxygen-18 isotopes of CO_2. Anal Chem. 2018;90:1384–1387.

[35] Lavecchia R, Zugaro M. Thermal denaturation of erythrocyte carbonic anhydrase. FEBS Lett. 1991;292:162–164.

[36] Effros RM, Taki K, Dodek P, et al. Exchange of labeled bicarbonate and carbon dioxide with erythrocytes suspended in an elutriator. J Appl Physiol. 1988;64:569–576.

Figure legends

Fig. 1. Time of equilibration of ^{18}O of water and CO_2. Water samples (DSW) with CA (solid line) as compared to that without CA (broken line). The $\delta^{18}O$ values (‰) are means ± SD *vs* V-SMOW (n=5). Note: For the x-axis non-linear time intervals were used.

Fig. 2. Time of equilibration of ^{18}O of water (DSW) and CO_2. Water sample (DSW) with CA in dependence on different treatments: fresh CA (full circles), CA defrosted from –20 °C after 14 d storage (full squares), sample treated with ultra sound for 5 min (full triangles), sample heated in the water bath at 50 °C for 15 min (open diamonds, broken line). The $\delta^{18}O$ values (‰) are means ± SD *vs* V-SMOW (n=5). Asterisks at samples points (broken line) means values are significantly different ($P<0.05$) from the averaged values of the other treatments. Note: see Figure 1.

Fig. 3. Time of equilibration of ^{18}O of water and CO_2. Plasma with CA (full triangles) and without CA (open triangles, broken line), whole blood fresh (crosses) and defrosted from –20 °C both without added CA (full diamonds). Plasma and blood samples are compared with water samples (DSW) + CA (full circles) and without CA (open circles, broken line). The $\delta^{18}O$ values (‰) are means ± SD *vs* V-SMOW (n=5). Note: see Figure 1.

Fig. 4. Time of equilibration of ^{18}O of water and CO_2 and ^{2}H of water and H_2. Water (DSW) samples without CA (^{18}O: open circles, broken line), with CA (^{18}O: full circles), with Pt (^{18}O: full squares, ^{2}H: open squares) and with CA+ Pt (^{18}O: crosses, broken line, ^{2}H: crosses, solid line). The δ values (‰) are means ± SD *vs* V-SMOW (n=5). Values at 1 h are not presented. Note: see Fig. 1.

Table 1. Studies of the effect of carbonic anhydrase (CA) fixed on glass beads on the ^{18}O and ^{2}H equilibration in water and blood samples.

Experiment	Sample			
1	DSW	DSW	DSW	DSW
^{18}O measurement	+ freshly prepared CA glass beads	+ CA glass beads defrosted from –20 °C after storage over 14 days (=CA-20)	+ CA-20 treated with ultrasound for 5 min.	+ CA-20 heated in the water bath at 50°C for 15 min.
2	blood plasma	whole blood (fresh)	whole blood (defrosted from –20 °C)	
^{18}O measurement	+ CA-20	no CA-20	no CA-20	
3	DSW	DSW	DSW	DSW
combined ^{18}O and ^{2}H measurement	no CA-20	+CA-20	+Pt	+CA-20+Pt

Figures 1- 4

Figure 1

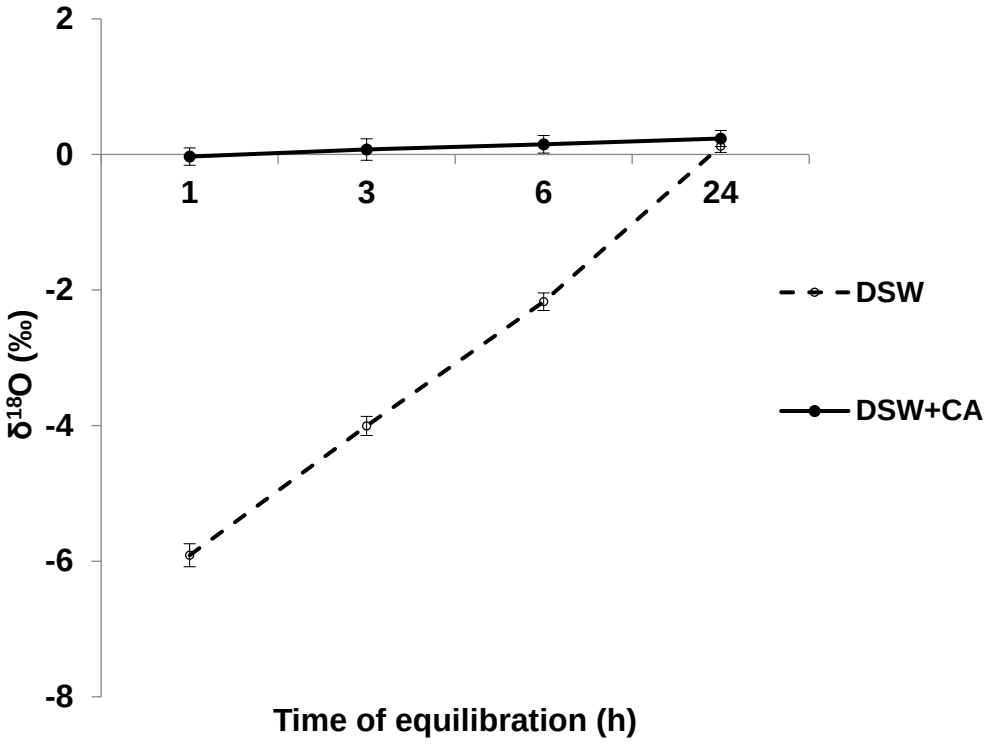

Figure 2

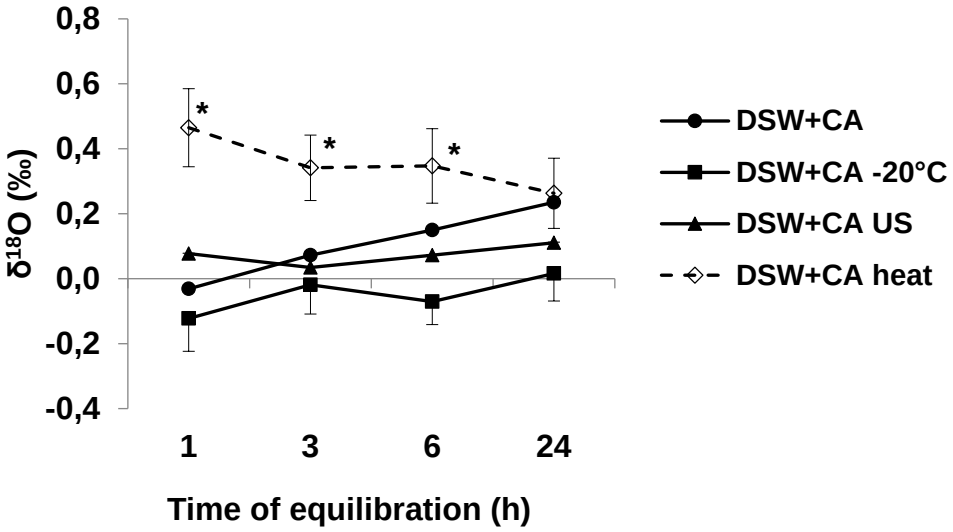

Figure 3

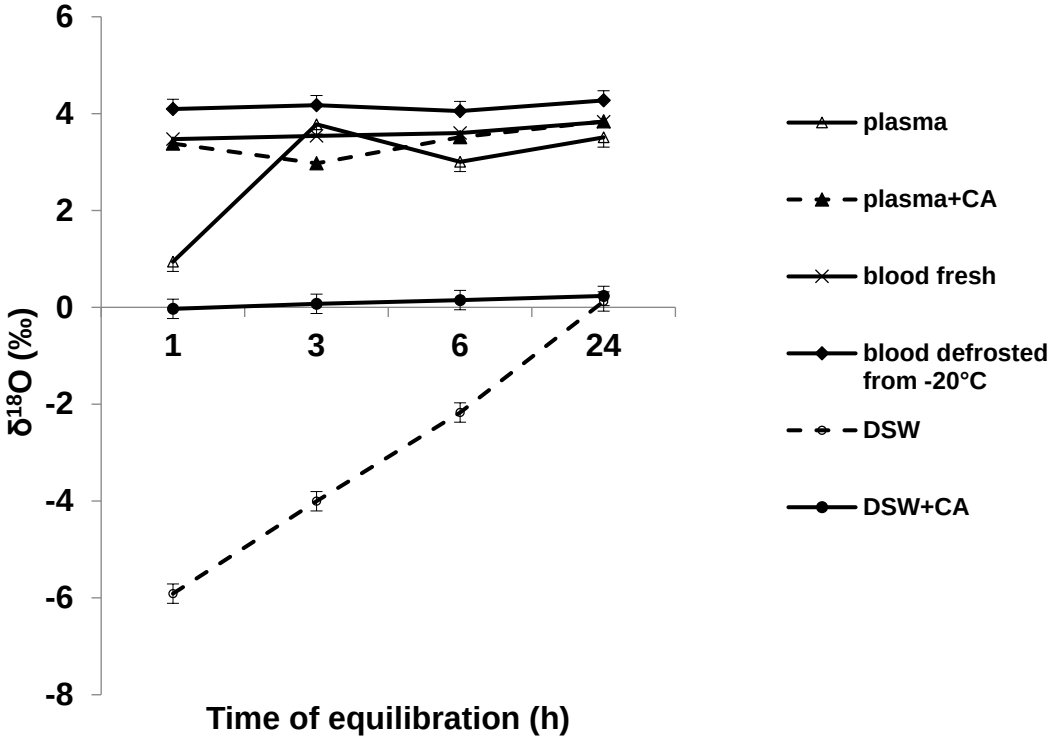

Figure 4