Preparation of hydrogen from water by reduction with lithium aluminium hydride for the analysis of $\delta^2$H by isotope ratio mass spectrometry

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An off-line technique is described for the preparation of $H_2$ from water prior to analysis of $\delta^2$H by dual-inlet isotope ratio mass spectrometry. $H_2$ is produced from sample water by reaction with LiAlH$_4$. This provides a rapid and inexpensive method for the analysis of $\delta^2$H in small (10 $\mu$L) samples of water. Precision was ±4.2 to 8.0 (1σ, n = 8) $\delta^2$H VSMOW for samples between 428 and 1500 $\delta^2$H VSMOW, ±14.5 $\delta^2$H VSMOW for water enriched to 3750 $\delta^2$H VSMOW and ±26.0 $\delta^2$H VSMOW for water enriched to 6100 $\delta^2$H VSMOW. Accuracy was ±1.1 to 4.2 $\delta^2$H VSMOW for water standards from natural abundance to 1000 $\delta^2$H VSMOW. Further disadvantages of both the uranium and zinc techniques have been used as reducing agents to generate $H_2$ from sample water. Both techniques are accurate and precise for samples with natural $\delta$H but have not been evaluated for high $\delta$H enrichment samples such as those produced in doubly labelled water studies of small animals. The levels of measurement precision of $\delta$H would contribute 2.6–3.8% to the precision error in estimates of small animal energy expenditure made using the doubly labelled water technique when duplicate analyses are performed.

$\delta$H VSMOW precisions for water enriched to 3750 to 6100 $\delta$H VSMOW are 4.2 to 8.0 (1σ, n = 8) $\delta$H VSMOW. For waters enriched to 1000 $\delta$H VSMOW the precision error is 26.0% for water standards from natural abundance to 1000 $\delta$H VSMOW. (The highest enrichment at which water of accepted $\delta^2$H is currently available). This method for $\delta^2$H determination is most appropriate for use with small (<50 $\mu$L) samples of high $\delta^2$H enrichment such as those produced in doubly labelled water studies of small animals. The levels of measurement precision of $\delta$H would contribute 2.6–3.8% to the precision error in estimates of small animal energy expenditure made using the doubly labelled water technique when duplicate analyses are performed. Copyright © 2000 John Wiley & Sons, Ltd.

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Measurement of the natural and tracer enrichments of $^2$H are used in studies of animal and human physiology to determine water turnover rate and body water content. Depletion rates of tracer levels of $^{18}$O and $^2$H are used to calculate the rate of CO$_2$ production and hence energy expenditure, by the doubly labelled water (DLW) technique. Recent expansion in the use of these techniques means that rapid, accurate and inexpensive methods of $\delta^2$H analysis are required for water from human or animal body fluids.

The $\delta^2$H of water in body fluid samples is normally determined by isotope ratio mass spectrometry following reduction to $H_2$ (reviewed by Wong and Klein). Two of the most common reduction techniques have been to generate $H_2$ by reaction of sample water with either uranium or zinc. Both of these techniques have disadvantages. The uranium technique involves passing each sample through a uranium furnace. This is time consuming and can produce memory effects in the results between samples. There are also potential hazards in handling uranium since it is an alpha particle emitter. The zinc technique produces accurate results for samples with natural $\delta^2$H, but accuracy declines as $\delta^2$H increases. Results obtained using zinc reduction may also vary between batches of the zinc reagent. A further disadvantage of both the uranium and zinc techniques is that the $H_2$ generated from the sample must normally be introduced through the manifold inlet ports of a mass spectrometer, so the number of samples processed daily is limited. These techniques also both require costly vacuum preparation lines. More recently, chromium and manganese have been used as reducing agents to generate $H_2$ from sample water. Both techniques are accurate and precise for samples with natural $\delta^2$H but have not been evaluated for high enrichment samples such as those produced in DLW studies.

Recent development of automated sample acquisition systems for isotope ratio mass spectrometers has prompted the use of new techniques for $\delta^2$H analysis of the water in body fluids. These automated preparation systems offer a large increase in the number of samples that can be processed daily, because batches of samples can be analysed when the mass spectrometer is not attended. Gas samples are typically drawn into the mass spectrometer from individual glass vials which contain $H_2$ produced by equilibration of sample water with $H_2$ of known $\delta^2$H in the presence of a platinum catalyst. Alternatively, acetylene can be produced by reaction of sample water with calcium carbide or $H_2$ can be produced by on-line pyrolysis of water in a nickel furnace. Each of these techniques has some practical limitations. Equilibration techniques require relatively large (100 $\mu$L to 5 mL) volumes of sample water. Such sample sizes are available in studies of humans or other large animals, but not in studies of small animals (where only 20–50 $\mu$L of water are normally available for analysis of $\delta$H). The accuracy of the calcium carbide technique is limited by variability in $\delta^{13}$C which interacts with the hydrogen signal. The pyrolysis technique requires purchase of additional equipment that is not currently available for all makes of mass spectrometer.

Analysis of $\delta^2$H in small water samples of high $\delta^2$H enrichment would be facilitated by an accurate, rapid and economical method of $H_2$ preparation that can be performed.
in individual vials from which gases can be drawn directly into a mass spectrometer by an automated gas acquisition system. We describe such a method, based on reduction of water to H₂ by LiAlH₄. This reaction has been used previously to generate H₂ in much larger volumes for analysis of δ²H by gas chromatography.

METHODS

Preparation of H₂ from water

H₂ is produced by reaction of water with an excess of LiAlH₄ powder (Aldrich, Poole, Dorset, UK) in red stopper (sterile, silicone-coated, no additive) 10 mL Vacutainers (Becton Dickinson, Meylan, Cedex, France). The reaction:

LiAlH₄ + 4H₂O → LiAl(OH)₄ + 4H₂

occurs instantly. A ten-fold excess of LiAlH₄ powder (0.04–0.05 g) is used to ensure that the entire 9–11 mL sample of water is reduced. The reaction produces approximately 0.05 g H₂ is produced by reaction of water with an excess of LiAlH₄ powder. Batches of 30–50 Vacutainers are prepared immediately before transfer to the mass spectrometer for analysis. Up to 200 samples may be prepared and analysed daily.

Material analysed

The δ²H was determined in seven water samples of known δ²H (five standards distributed by the International Atomic Energy Authority (IAEA, Vienna, Austria) and two of the Boston standards defined in Ref. 21) and four water samples (coded A to D) enriched to unknown δ²H (range: natural abundance ca. 6000‰). The δ²H of samples A to D was designed to cover the range encountered in samples derived from studies of small animals, since δ²H in such samples is often higher than the enrichments of the standards of known δ²H. Eight independent replicate analyses were performed for all samples for which data are reported here. We normally carry out duplicate analyses on body water samples collected from small animals. Further samples are analysed if the coefficient of variation (CV, defined as [standard deviation × 100]/mean) of the calculated ppm of the sample exceeds 2%. The CV for duplicate analyses of water distilled from blood from small animals is typically 0.2–0.5%.

Normalisation of δ²H to the Standard Light Antarctic Precipitation-Vienna Standard Mean Ocean Water (SLAP-VSMOW) scale

Isotope enrichments are defined as δ²H VSMOW = ([²H/H]Sample/[²H/H VSMOW]) – 1) × 1000. The δ²H relative to reference gas (δ²H REF) of the sample of H₂ generated from water using LiAlH₄ that is measured by the mass spectrometer requires normalisation to be consistent with the SLAP-VSMOW scale for two reasons. First, half the atoms in the H₂ analysed originate from sample water and half from the LiAlH₄ reagent. The δ²H of the H₂ analysed is therefore approximately half that in the sample water due to the contribution of the hydrogen of background δ²H from the LiAlH₄. Second, δ²H REF is measured relative to our laboratory standard reference gas with an enrichment of 10 δ²H VSMOW (rather than relative to VSMOW with δ²H = 0). Both these effects are corrected for using the δ²H REF of triplicate analyses of three laboratory working standards (coded A, B and D in Table 1) which are prepared with each
batch of 30–50 samples and are run on the mass spectrometer immediately prior to the samples. We use least-squares regression to calculate the relationship between $\delta^2$H$_{\text{REF}}$ for the three working standards and $\delta^2$H$_{\text{VSMOW}}$ for these standards for each batch of samples. The R$^2$ value for this relationship is always 100% and the P value <0.001. The regression equation is rearranged to calculate $\delta^2$H$_{\text{VSMOW}}$ for each sample. The $\delta^2$H$_{\text{VSMOW}}$ of the laboratory working standards was determined from the mean values predicted by running them immediately prior to the standards of known enrichment (shown in Table 1) on 6 days. The $\delta^2$H$_{\text{VSMOW}}$ of the laboratory working standards are checked every 2–6 months against the known standards; these have remained unchanged for 2 years.

Mass spectrometry

A Micromass (Altrincham, Cheshire, UK) Multiprep system was used to transfer the sample gas from each Vacutainer to a Flexi-cool water trap (−100°C) and then to a dual-inlet system where the sample and reference gas were balanced prior to analysis by an isotope ratio mass spectrometer (Optima, Micromass).

Definitions of precision and accuracy

We use the standard deviation (SD, $\sigma_n$) of replicate analyses of each sample performed in separate Vacutainers (n = 8) to quantify precision. We define accuracy as the difference between the mean $\delta^2$H$_{\text{VSMOW}}$ determined by LiAlH$_4$ reduction and the mean accepted value for each standard.

RESULTS

Natural and low enrichment samples

The $\delta^2$H in natural and low enrichment (up to ca. 1500 $\delta^2$H$_{\text{VSMOW}}$) samples was measured with a precision of between 1.9 and 3.6‰ $\delta^2$H$_{\text{REF}}$ across 8 replicate analyses (Table 1). Since half the atoms in the H$_2$ originated from the LiAlH$_4$ reagent and half from the sample water, normalisation of results to the SLAP-VSMOW scale involved approximately 2-fold expansion of scale from the delta values expressed relative to the reference gas. This reduced the precision of the normalised delta values to 4.2–8.0‰ $\delta^2$H$_{\text{VSMOW}}$ (Table 1). Accuracy was 1.0–4.2 $\delta^2$H$_{\text{VSMOW}}$ (Table 1) across the range of enrichments (−428 to 996 $\delta^2$H$_{\text{VSMOW}}$) for which accepted water standards are available.

High enrichment samples

Precision was 6.1‰ $\delta^2$H$_{\text{REF}}$ relative to the reference gas for samples enriched to ca. 3750 $\delta^2$H$_{\text{VSMOW}}$ and 11.4‰ $\delta^2$H$_{\text{REF}}$ relative to reference gas for samples enriched to ca. 6100 $\delta^2$H$_{\text{VSMOW}}$ (Table 1). Normalisation of results to the SLAP-VSMOW scale reduced precision to 14.5‰ $\delta^2$H$_{\text{VSMOW}}$ for water enriched to ca. 3750 $\delta^2$H$_{\text{VSMOW}}$ and 26.0‰ $\delta^2$H$_{\text{VSMOW}}$ for water enriched to ca. 6100 $\delta^2$H$_{\text{VSMOW}}$.

DISCUSSION

Precision of analyses

Measurement precision for the standards Boston 1 and 3 obtained in the current study is in the middle of the range of 18 laboratories which analysed these standards.21 Most of these laboratories used zinc or uranium reduction to determine $\delta^2$H$_{\text{VSMOW}}$. Precision for samples enriched to the levels used in studies of humans (up to ca. 1000 $\delta^2$H$_{\text{VSMOW}}$) is better (±4–8 $\delta^2$H$_{\text{VSMOW}}$ in the current study) than the ±10 $\delta^2$H$_{\text{VSMOW}}$ reported in studies which performed analyses using zinc.7 Precision of analyses of standards IAEA 302a and 302b in the current study is within the range (up to ±8.6 $\delta^2$H$_{\text{VSMOW}}$) found by 11 laboratories using zinc.22 Results obtained using LiAlH$_4$ are therefore more precise than those normally obtained using zinc, especially for more enriched samples.

Precision of analyses of $\delta^2$H$_{\text{VSMOW}}$ of water by LiAlH$_4$ reduction (±1.9–3.6 $\delta^2$H$_{\text{VSMOW}}$) is slightly lower than that reported for natural abundance and low enrichment water by equilibration (±0.1–3.7 $\delta^2$H$_{\text{VSMOW}}$).10,14,42 Analysis by on-line pyrolysis and chromium or manganese reduction also produces more precise results for natural abundance water (±0.8–4 $\delta^2$H$_{\text{VSMOW}}$).11,12,17,18 Precision of analyses performed by pyrolysis is similar to that reported here using LiAlH$_4$ at higher enrichments (±9δ at 1200 $\delta^2$H$_{\text{VSMOW}}$).18 Equilibration, pyrolysis and chromium or manganese reduction therefore normally produce more precise results than reduction with LiAlH$_4$ for low enrichment samples. However, equilibration requires 0.25–1 mL of sample water and therefore cannot be used with the 20–50 µL samples normally available from physiological studies of small animals.

Measurement precision of $\delta^2$H$_{\text{REF}}$ in H$_2$ produced from water using LiAlH$_4$ was as good as by pyrolysis or hydrogen equilibration when results were expressed relative to the working standard (Table 1); reduction in precision to below that of these techniques for low enrichment sample water occurred due to expansion of results to the SLAP-VSMOW scale. Although expansion of results to the SLAP-VSMOW scale is a disadvantage of the technique since it decreases measurement precision, derivation of half the H$_2$ from the LiAlH$_4$ could increase the accuracy of results generated using LiAlH$_4$ for highly enriched samples in comparison with techniques in which hydrogen from sample water alone is used to generate the H$_2$ analysed. This is because mass spectrometer machine precision becomes lower as the abundance of the rare isotope in the sample gas rises above a critical level. This aspect of the LiAlH$_4$ technique is a particular benefit when determining $\delta^2$H in body fluid samples from small animals since the appropriate labelling isotope enrichments for measurement of CO$_2$ production by the DLW technique increase as body mass declines.5
available for natural abundance samples and more accurate than alternative techniques as $\delta^2H$ increases to approximately 1000 $\delta^2H_{\text{VSMOW}}$. No water standards of agreed $\delta^2H$ greater than 1000 $\delta^2H_{\text{VSMOW}}$ are currently available, so the accuracy of analyses cannot be determined at higher enrichments.

Influence of precision of $\delta^2H$ analyses on the precision of daily energy expenditure calculated by the doubly labelled water technique

The DLW technique allows calculation of the rate of CO$_2$ production of a subject from the elimination rates of a dose of water enriched with $^{18}$O and $^2$H. The $^2$O isotope is eliminated from the body in CO$_2$ and water whilst $^2$H is eliminated only in water. The difference in turnover rates is used to calculate CO$_2$ production and hence energy expenditure. We carried out a simulation to evaluate the contribution that the observed levels of precision in determination of $\delta^2H_{\text{VSMOW}}$ using LiAlH$_4$ would make to the error in energy expenditure estimated by the DLW technique. We based the simulation on empirical data generated in a DLW experiment from two laboratory mice. One mouse was a non-reproductive individual with a high elimination rate ratio ($k_o/k_d$) of 1.83 (where $k_o$ and $k_d$ are the rates of loss from the animal of $^{18}$O and $^2$H, respectively). The other animal was lactating and had a low elimination rate ratio of 1.26. Error attributable to precision in $^2$H isotope analyses is expected to increase as the isotope elimination ratio decreases.

For each individual mouse we used the empirically observed mean $\delta^{18}$O$_{\text{VSMOW}}$ and the $\delta^2H_{\text{VSMOW}}$ at the background level before the animals were labelled, 1 h after an injection of water enriched with $^2$H and $^{18}$O (initial sample) and at the end of the 24 h experiment (final sample). Mean background, initial and final $\delta^2H_{\text{VSMOW}}$ were ~32, 2567 and 2034% for the non-reproductive mouse and ~6, 1624 and 622% for the lactating mouse. The predicted variation (SD) around each mean $\delta^2H_{\text{VSMOW}}$ was calculated from the least-squares regression equation which describes the relationship between SD and $\delta^2H$ enrichment (SD = 0.30030(0.0003) $\times \delta^2H_{\text{VSMOW}}$ + 5.2478(0.7338), SD in brackets, $R^2 = 0.90$, P < 0.001, n = 11; calculated from data in Table 1). We used the random number generator in Minitab$^9$ (DEC Corp., Pennsylvania, USA) to generate simulated $\delta^2H_{\text{VSMOW}}$ data which was distributed as standard normal deviates around the mean values using the standard deviation predicted for each enrichment. We generated duplicate data from each distribution and entered these data along with the fixed $\delta^18$O into a dedicated computer package$^{24}$ for calculation of CO$_2$ production and energy expenditure from the DLW technique. We repeated the process 25 times for each animal to generate distributions of energy expenditure estimates. Because $\delta^{18}$O$_{\text{VSMOW}}$ at background, initial and final sampling times was fixed, the variation in energy expenditure in these simulations is due to precision error in mean $\delta^2H_{\text{VSMOW}}$ from duplicate analyses using LiAlH$_4$. The mean energy expenditure across the 25 simulations was 58.4 kJ day$^{-1}$ (SD = 1.5; CV = 2.6%) for the non-reproductive mouse and 149.1 kJ day$^{-1}$ (SD = 5.6; CV 3.8%) for the lactating mouse.

This simulation indicates that when duplicate analyses of $\delta^2H$ are made at background, initial and final time points using LiAlH$_4$, the precision error in the $\delta^2H$ analyses would generate between 2.6 and 3.8% error in the CO$_2$ production and energy expenditure estimates, depending on the $k_o/k_d$ ratio. For comparison, discrepancies between DLW estimates of CO$_2$ production and estimates based on indirect calorimetry, ignoring the sign of the difference ($=\text{mean deviation}$), average about 10–15%.$^5$

Recommendation for use of LiAlH$_4$ reduction to determine $\delta^2H$ in water

Reduction of sample water with LiAlH$_4$ produces results that are more accurate and more precise than those normally obtained by zinc or uranium reduction. Analysis of $\delta^2H_{\text{VSMOW}}$ in water by reduction with LiAlH$_4$ is therefore preferable to using zinc or uranium. Analyses of $\delta^2H$ in water with LiAlH$_4$ are as accurate as those performed by equilibration, pyrolysis and chromium or manganese reduction for natural abundance samples and can be more accurate for enriched samples. However, precision of analyses is normally greater when analyses are performed by equilibration, pyrolysis, chromium or manganese reduction, especially for low enrichment samples. An equilibration technique may therefore be the preferred method of $\delta^2H$ determination for large (0.25–1 mL) water samples. Pyrolysis would be the best method to use with very small (<1 µL) natural or low abundance samples. Reduction with LiAlH$_4$ is most appropriate for $\delta^2H$ determination in water samples such as those from physiological studies of small animals, which are typically highly enriched with $^2$H and of which only 20–50 µL of sample are available.

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REFERENCES