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Application of Gas Chromatography-Tandem Mass Spectrometry (GC/MS/MS) for the Analysis of Deuterium Enrichment of Water

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Abstract

Incorporation of deuterium from deuterium oxide ($^2\text{H}_2\text{O}$) into biological components is a commonly used approach in metabolic studies. Determining the dilution of deuterium in the body water pool (BW) can be used to estimate body composition.

We describe three sensitive GC-MS/MS methods to measure water enrichment in BW. Samples were reacted with NaOH and $\text{U-}^{13}\text{C}_3$ -acetone in an autosampler vial to promote deuterium exchange with $\text{U-}^{13}\text{C}_3$ -acetone hydrogens. Headspace injections were made of $\text{U-}^{13}\text{C}_3$ -acetone-saturated air onto a 30m DB-1MS column in EI-mode.

Subjects ingested 30ml $^2\text{H}_2\text{O}$ and plasma samples were collected. BW was determined by standard equation. DXA scans were performed to calculate body mass, body volume and bone mineral content. A 4 compartmental model was used to estimate body composition (fat and fat free mass).

Full scan experiments generated a m/z 45 peak and to a lesser extent a m/z 61 peak. Product fragment ions further monitored included 45 and 46 using selected ion monitoring (SIM;Method1), the $61>45$ and $62>46$ transition using multiple reaction monitoring (MRM;Method2) and the Neutral Loss, $62>45$, transition (Method3). MRM methods were optimized for collision energy (CE) and collision-induced dissociation (CID) argon gas pressure with 6eV CE and 1.5 mTorr CID gas being optimal. Method2 was used for finally determination of $^2\text{H}_2\text{O}$ enrichment of subjects due to lower natural background.

We have developed a sensitive method to determine $^2\text{H}_2\text{O}$ enrichment in body water to enable measurement of FM and FFM.

Keywords

acetone; body composition; deuterium; gas chromatography; tandem mass spectrometry

Introduction

Measuring the incorporation of deuterium (^2H) from deuterated water ($^2\text{H}_2\text{O}$ or D_2O) into biological components is a commonly used tool in metabolic studies to determine body water turnover (Wolfe et al., 2005; Westerterp, 1999) and the synthesis rates of certain molecules (Wolfe et al., 2005; Holm et al., 2013; Delgado-Lista et al., 2013; Robinson et al., 2011; Gasier et al., 2009; Cabral et al., 2008; Letexier et al., 2003). For instance after intake of $^2\text{H}_2\text{O}$, glucose, fatty acids, cholesterol, amino acids, nucleotides and other molecules of metabolic interest become enriched with deuterium from the $^2\text{H}_2\text{O}$ as it exchanges with hydrogens in C-H bonds through associated enzymatic reactions generating a C-D bond.

^2H dilution in body water is also used to determine body composition (Westerterp, 1999; Wilson et al., 2013; McCabe et al., 2006). A known quantity of $^2\text{H}_2\text{O}$ is administered and, after deuterium equilibration, the enrichment of deuterium in body water is measured. Fat-free mass is then determined based on the calculation of total body water (TBW) divided by 0.73, where 0.73 represents 730g H_2O /kg fat-free mass (73%) (Westerterp, 1999; Lee et al., 2008; Wang et al., 1999). Subsequently, fat mass is calculated by subtracting the fat-free mass from the total body mass. Body composition measurements are an essential tool in many clinical nutrition studies and need to be applied in large groups of subjects.

The use of a 4-compartmental model for body composition determination in which dual-energy x-ray absorptiometry (DXA) is used to estimate bone mass and volume and $^2\text{H}_2\text{O}$ dilution to estimate fat-free mass has become accepted as a gold standard as it reduces the variability of each compartment and therefore improves the accuracy of body composition estimates (Wilson et al., 2013). This approach requires the development of an easy applicable and cheap method to estimate the ^2H enrichment of body water.

Many approaches for determining deuterium enrichment in water have been reported and include its assessment by conversion of the hydrogen and deuterium of water to gas with analysis done on an expensive isotope ratio mass spectrometry (IRMS) (Hachey et al., 1987; Scrimgeour et al., 1993), by ^2H exchange into acetylene analyzed by IRMS (Van Kreel et al., 1996) or GC-MS (Previs et al., 1996), and by ^2H exchange into acetone analyzed by GC-MS (Yang et al., 1998). Although these methods are effective and reliable, the IRMS based methods are labor intensive, which can limit throughput. Recently, a method was developed that is simple, inexpensive, and decreases sample preparation and instrument runtime (Shah et al., 2010). In this approach, sample (plasma, urine, tears, etc.), sodium hydroxide, and acetone are added directly to a GC vial and are allowed to react prior to analysis. In the presence of sodium hydroxide, deuterium from the water in the sample non-enzymatically exchanges with the hydrogens in acetone. Subsequently, the acetone in the vial headspace is injected into the GC and analyzed. The major disadvantage of this method is that it does not distinguish a deuterium-derived unit increase in the mass of the analytical reporter molecule from one derived from its natural enrichment within acetone of ^{13}C (~3%): a true $^2\text{H}_2\text{O}$ enrichment manifests therefore will lead only to a small increase over this non-zero background. This problem can be solved by using Acetone ($\text{U-}^{13}\text{C}_3$) (Yang et al., 1998).

Here, we report the development, validation, and application of a new GC-MS and GC tandem MS (GC-MS/MS) method for the determination of very low stable isotopic enrichments of deuterium in the water of biological samples using the acetone ($\text{U-}^{13}\text{C}_3$) headspace method.

Materials and Methods

Materials

All solvents were GC-MS grade, purchased from Sigma-Aldrich. Sodium hydroxide was from VWR. Acetone ($\text{U-}^{13}\text{C}_3$) and deuterated water were purchased from Sigma Isotec.

Sample Preparation

After sampling, immediately transfer blood into lithium-heparin or EDTA containing blood tubes, centrifuge at $8,000 \times g$ for 5 min to yield plasma, and transfer an aliquot into a 1.5mL Eppendorf tube. For urine samples, after thorough mixing, place an aliquot into a 1.5mL Eppendorf tube. Plasma and aliquoted urine can be used for immediate analysis or stored at -80°C for later analysis. For sample preparation for GC-MS or GC-MS/MS analysis, 10 μL of sample, 2 μL of 1 M sodium hydroxide, and 5 μL of $\text{U-}^{13}\text{C}$ -acetone were added to a GC vial with threaded opening. The GC vial was then closed with a septum cap of a type previously determined to form a gas-tight seal, then incubated for 24 hours at room temperature prior to GC-MS or GC-MS/MS analysis.

Gas chromatography – tandem mass spectrometry (GC-MS/MS)

Analyses were carried out on a Bruker GC-MS/MS system consisting of a Scion TQ triple quadrupole mass spectrometer, a 436-GC gas chromatograph with Programmable Temperature Vaporization (PTV) inlet, and a CP-8400 autosampler. The GC was equipped with a 30 m DB-1MS capillary column (0.25 mm i.d., 0.25 μm film thickness, Agilent). Helium was used as the carrier gas at a rate of 1 mL min^{-1} . One μL of vial headspace gas were autosampled and injected into the PTV inlet equipped with a Sky 2.0 mm ID Top Taper Inlet Liner and set to a 20:1 split ratio with the inlet temperature set at 220°C . An isothermal oven temperature program was used and set at 190°C for 2.5 min. Both the transfer line and the electron impact (EI) source of the Scion TQ were set to 150°C . The source filament voltage was 70eV. To develop the method, collision cell argon gas pressure was set to 0.2, 0.6, 1.0, 1.5, and 2.0 mTorr and with a range of CID energies from 0 to 50 eV. Acetone ($\text{U-}^{13}\text{C}_3$) and acetone (^2H , $\text{U-}^{13}\text{C}_3$) were detected by selected ion monitoring (SIM:Method1) of the in-source generated m/z 45 and 46 ions, respectively, and by multiple reaction monitoring (MRM) of the m/z 61 to 45 and 62 to 46 transitions (Method2) and summed m/z 62 to 46 and 62 to 45 transitions (Method3).

Clinical Experiment

Healthy young ($n=4$) and older ($n=6$) adults 20–79 years of age were used in this study and are a subset of a larger study. A background blood sample was collected prior to ingesting 30 mL of deuterium oxide. The use of 30 mL of deuterium oxide is calculated to enrich total body water of subjects by 0.006 to 0.01%. Blood samples were subsequently collected every hour for a total of seven hours. Subjects started the study fasted and laid supine for the

duration of the study. As part of the larger study, subjects consumed a liquid high protein meal at hour 4. The ^2H -enrichment of plasma water was measured by GC-MS/MS and by GC-MS using $\text{U-}^{13}\text{C}$ -acetone. For 4-compartmental calculations of body composition, the median plasma ^2H -enrichment of hours 2 through 7 were used. The equations used for calculating total body water and FM and FFM using the Lohman's 4C body composition model are as previously described (Wilson et al., 2013).

Results and Discussion

In this study, we set out to develop and validate a novel yet simplified method using GC tandem mass spectrometry, in addition to the commonly used GC-MS to determine the enrichment of deuterium in biological samples. Processing samples using this protocol is rapid and consist of reacting a biological sample (urine, plasma, saliva) with acetone (and in our case uniformly ^{13}C labeled acetone) under alkaline conditions directly in the autosampler vial as previously reported by (Shah et al., 2010). Under these conditions, the deuterium in the samples exchanges with the hydrogens in acetone. Acetone has 6 exchangeable positions for deuterium (Figure 2). In two of our methods we fragment $\text{U-}^{13}\text{C}$ -acetone in Q2 while the first method fragments $\text{U-}^{13}\text{C}$ -acetone in the source, leading to all methods reducing the number of hydrogen positions to three that could contain an exchanged deuterium. Others have shown that this reaction is complete in less than 5 hours (Yang et al., 1998); however we allowed the reaction to occur for 24 hours to ensure complete deuterium/hydrogen exchange.

Sample Preparation and Method Development

When using the reaction mixture ratio 2/1/2 of sample:NaOH: $\text{U-}^{13}\text{C}$ -acetone, it was demonstrated (Shah et al., 2010) that when injecting 5 μL of headspace into the GC the subsequent injection of headspace from the same vial showed a 50% reduction in signal intensity. We show, however, that using a reaction mixture ratio of 5/1/2.5 and injecting only 1 μL of headspace signal intensity is maintained for 10 injections with a coefficient of variation of 2.6%. This reproducibility is maintained for 48 hours after samples are initially prepared. Therefore, under these conditions, acetone stability in the autosampler vial is maintained during the course of the 5 injections that we use for each standard/sample.

With this simplified method of processing and reacting samples directly in the autosampler vial, we eliminate solvent extraction steps, which favors higher precision. The use of headspace injection eliminates the need to modify the analytical conditions to remove a solvent interference peak, both advantages as reported by Yang et al. (1998). In a preliminary experiment, standards and plasma from humans were incubated with NaOH, but without acetone ($\text{U-}^{13}\text{C}$) to ensure lack of substantial background noise and the lack of peaks interfering with either of our three methods. To avoid cross-contamination between injections, the gas-tight syringe is rinsed with air before and after each injection. Between each sample run, a wash is included by injecting air and programming the column oven temperature to hold at 190°C for 1.5 min, then ramp to 320°C at a rate of $120^\circ\text{C}/\text{min}$ with no hold time, and then ramp down to 190°C with a total run time of 3.67 min.

Injection of U-¹³C-acetone saturated headspace gas onto the GC yields two spectral peaks (45 and 61 *m/z*) on a full-scan single-quadrupole mode spectrum acquired during elution of a major GC peak (Figure 1A). Mass chromatograms showed a single peak substantially free of baseline noise during selected ion monitoring (SIM) of *m/z* 45 (Figure 1B) and *m/z* 61 (Figure 1C). After optimization of MS voltage and gas settings to maximize *m/z* 61 ion transmittance, that ion was subjected to fragmentation in Q₂ of the MS/MS operated in product scan mode. Fragments generated from fragmentation in Q₂ were 45.1 and to a lesser extent, 61.1 (Figure 3). Fragmentation of the 61.1 ion in Q₂ was optimized by conducting experiments while varying the collision energy (CE) and collision-induced dissociation (CID) gas pressure. As shown in Figure 4, a CE of 6 eV and a CID gas pressure of 1.5 mTorr were determined to be optimal in generating the *m/z* 45 fragment.

Fragmentation

The symmetrical molecule U-¹³C-acetone can fragment to lose either of the two methyl groups, which both generate a *m/z* 45 fragment ion (Figure 2). Depending on the location of a deuterium acquired from alkali-catalyzed exchange from ²H₂O, it can either be retained in the charged deprotonated acetaldehyde product (making it *m/z* 46) or lost to the neutral (therefore undetected) methyl radical, with no change to the *m/z* 45 ion. Three possible methods could be used based on these findings. One that looks for the retention of ²H, yielding from a *m/z* 62 precursor a *m/z* 46 ion; one that indirectly looks for its neutral loss, yielding from the *m/z* 62 precursor a *m/z* 45 ion; and one that sums intensities in the channels monitoring ²H retention and ²H loss. If acetone were used as the analytical reporter (Yang et al., 1998; Shah et al., 2010), then the analogous neutral loss method 59:43 would offer the clear advantage that the label-bearing fragment has but one carbon instead of two, thus roughly halving the expected background from natural ¹³C. Our use of uniformly enriched acetone (U-¹³C₃) negates this advantage, leaving the 62:46 direct method with a technical advantage: at settings of 62 and 46, both quadrupole mass filters select against crosstalk from the vast excess of U-¹³C₃ acetone not bearing deuterium, detected by the 61:45 transition. Each has its pros and cons. Transition of 61 to the 45 ion in Q₂ is optimal due to the 45 fragment containing 1 less carbon and therefore, lowers the natural abundance.

Natural Abundance

Overall, the use of U-¹³C₃-acetone greatly reduces the natural isotopic background compared to acetone with natural abundance of ¹²C (Fig. 5). In experiments with unlabeled acetone, we observed a natural abundance of 2.86% when monitoring peaks 43 and 44 using SIM (Method1), 2.56% when monitoring the transitions of 58>43 and 59>44 using MRM (Method2), and 3.85% when monitoring the transitions of 58>43, 59>43, and 59>44 using MRM (Method3). When using U-¹³C-acetone, the natural abundance when monitoring peaks 45 and 46 using SIM is 0.24% (Method1), when monitoring the transitions of 61>45 and 62>46 using MRM is 0.20% (Method2), and when monitoring the transitions of 61>45, 62>45, and 62>46 using MRM is 0.32% (Method3). This remaining background is explainable only in small part by the natural occurrence of isotopes of hydrogen and oxygen, and is likely caused in large part by crosstalk detection of the 61 mass in the 62 channel in Q1 for Method2 and Method3 or in the case of Method1, crosstalk detection of the 45 mass

in the 46 channel in Q1. This is due to the enormity of the 61 (or 45) peak when scanning in Q1 and the inaccessibility of mass resolution adjustment for Q1 on the Bruker TQ.

Calibration Curves and Limits of Quantitation

Linear calibration curves of ^2H -enrichment, in the 0 to 1% range, were acquired with slopes of 1.88, 2.17, and 3.86 for Method1, Method2, and Method3 (Figure 6). In addition, linear calibration curves of ^2H -enrichment, in a lower range of 0 to 0.1%, were obtained with slopes of 1.83, 2.20, and 4.0 for methods Method1, Method2, and Method3, respectively. The peak-area-ratio vs. known enrichment relationship was linear (typical $R^2 > 0.997$). Due to the lower M1/M0 natural abundance of the $\text{U-}^{13}\text{C}$ -acetone using Method2 (0.2% observed; 0.0767% expected), the limits of quantitation of ^2H -enrichment of water were $< 0.001\%$, which is well below the natural abundance of ^2H .

Body Composition

We further used these methods to determine deuterium enrichment in plasma samples collected from subjects that ingested deuterium oxide and combined with DXA data, can be used in a Lohman's 4C body composition model to more precisely estimate body composition. Additionally, the D_2O dilution approach can be used by determining the deuterium enrichment, which allows for total body water (TBW) calculation and subsequently FFM can be calculated by the following equation: $\text{TBW}/0.732$ where 0.732 is the hydration index of FFM. Body composition data generated by DXA, 4C model, and D_2O dilution for the ten subjects in this study are presented in Table 1. A high correlation was demonstrated for FM between DXA and 4C ($r\text{-squared}=0.9557$, $\text{slope}=1.179$, $y\text{-intercept}=-2.848$) and to a lesser extent, D_2O dilution ($r\text{-squared}=0.8659$, $\text{slope}=0.1.179$, $y\text{-intercept}=-4.821$). For FFM, a high correlation was demonstrated for between DXA and 4C ($r\text{-squared}=0.9559$, $\text{slope}=1.076$, $\text{intercept}=-5.131$) and to a lesser extent, D_2O dilution ($r\text{-squared}=0.9087$, $\text{slope}=0.1.141$, $y\text{-intercept}=-6.791$). The 4C model is considered the "gold standard" and independently assesses body density, body water, and bone. For body density, this approach is simpler due to the use of DXA data to calculate body volume, which eliminates the need for separate body volume devices. Further, the 4C model accounts for the individual components of FFM (aqueous and bone) rather than using a constant density and hydration (Fields et al., 2000). Taken together, our data show a high correlation of the Lohman's 4C body composition model and D_2O dilution with DXA.

Conclusions

Herein, we describe 3 methods to determine deuterium enrichment of biological samples. With the development of these 3 methods, deuterium enrichment determination can be obtained using either a GC-MS or a GC-MS/MS. Furthermore, by measuring deuterium enrichment, FM and FFM can be determined using the D_2O dilution technique by first determining TBW. In addition, this method can be utilized in a Lohman's 4C body composition model to more accurately determine body composition that only requires a DXA scan and TBW measurement.

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Figure 1.

Spectrum A was acquired 1.34 min post-injection of a $^{13}\text{C}_3$ -acetone headspace standard onto the GC. It features prominent m/z 45 and m/z 61 species. Chromatographs B and C show results of selected-ion monitoring of m/z 45 and 61 ions, respectively.

Figure 2.

This figure illustrates the deuterium exchange from deuterated water with the acetone hydrogens and the proposed collisional breakage of a m/z 61 $^{13}\text{C}_3$ - acetone radical cation that yields a m/z 45 product ion and the neutral methyl radical.

Figure 3. Using product scan mode, the precursor m/z 61 ion is fragmented in Q2 generating a m/z 45 fragment, which lowers the background due to the presence of less carbons.

Figure 4.
Optimization of fragmentation in Q2 using collision energy and CID gas pressure.

Figure 5. ^2H background abundance when using $\text{U-}^{13}\text{C}$ -acetone versus ^2H background abundance of unlabeled acetone Method1, Method2, and Method3.

Figure 6. Standard curve of ^2H -enrichment of water measured after hydrogen/deuterium exchange with $\text{U-}^{13}\text{C}$ -acetone using Method1, Method2, and Method3. The inset shows the plot from 0.005 to 1.0% ^2H -mol fraction.

Table 1
Body composition data of subjects generated by DXA, D₂O dilution, and 4C modeling

Subject	Body Mass	D ₂ O Ingested	Corrected ² H enrichment	TBW	DXA FM	D ₂ O FM	4C FM	DXA FFM	D ₂ O FFM	4C FFM
	kg	mL	TTR	L	kg	kg	kg	kg	kg	kg
A	73.6	30.03	0.000990	29.15	31.44	33.8	35.03	42.16	39.83	38.57
B	72.5	29.99	0.000707	40.81	20.50	16.8	18.51	52.00	55.75	53.99
C	63.3	30.00	0.001121	25.73	25.54	28.1	28.87	37.75	35.16	34.43
D	97.7	30.00	0.000575	50.21	32.89	29.10	34.03	64.83	68.59	63.69
E	89.8	30.02	0.000658	43.84	25.35	29.90	29.39	64.41	59.89	60.37
F	73.1	30.00	0.000681	42.36	12.39	15.20	14.94	60.71	57.87	58.15
G	76.3	30.00	0.000545	52.96	11.45	3.90	8.69	64.84	72.35	67.61
H	67.5	29.90	0.000706	40.71	14.08	11.90	14.15	53.39	55.62	53.32
I	86.7	30.00	0.000572	50.47	20.39	17.70	20.43	66.31	68.94	66.26
J	68.1	30.03	0.000685	42.14	13.87	10.5	12.63	54.23	57.57	55.47
Mean				41.84	20.79	19.69	21.67	56.06	57.16	55.19
Standard Deviation				8.79	7.85	9.94	9.46	10.03	12.00	11.04
CV, %				21.01	37.74	50.49	43.68	17.89	21.00	20.00