SOP: CASE MMPC

Heavy Water Assays:

$^{2}H_2O$ and $H_2{}^{18}O$

by GC-mass spectrometry

#CA2000 / CA2011 / CA2016A

Also needed for #CA2016 / CA2017

Summary:

Heavy water can be used as a tracer for estimating metabolic rates (*in vivo*, *in vitro*) such as total energy expenditure (TEE) and fractional synthesis rates (FSR; eg. protein, lipids, triglycerides). When using both $^{2}H_2O$ and $H_2{}^{18}O$ (DLW), TEE is estimated from the total production of CO_2 as measured by the differences in decay rates of labeled the ^{18}O and ^{2}H in body water over time following a single bolus of DLW (1). $^{2}H_2O$ can be used to estimate fractional synthesis rates of metabolic reactions such as those associated with proteins, lipids, triglycerides, and cholesterol (2,4).

Reagents and Materials:

Reagent/Material	Quantity Required	Vendor
Deuterium oxide, $^{2}H_2O$ (99 atom % excess);		Sigma-Aldrich
NaOH	2µL	stock
$H_2{}^{18}O$ (95% atom excess)		Isotec (Miamisburg, OH)
acetone/acetonitrile solution	4µL	stock
Chloroform	200- 500 µL	stock
Phosphorus Pentachlorate; TMP*	3 mg	stock
Diethyl ether	120µl	stock

Analysis of $^{2}H_2O$ and $H_2{}^{18}O$: ^{2}H labeling of body water is determined by exchange with acetone and the ^{18}O labeling of body water is determined by conversion to trimethyl phosphate *(TMP; generated by reacting phosphoric acid with diazomethane).

Protocols:

1. Quantitation by $^{2}H_2O$ Standard Curve:
2. Standards are made from deuterium oxide (Aldrich 617385)

3. Pipette 10µl of plasma or standard into Eppendorf

4. $^{2}H_2O$ standards
    a. Blank (MQ H_2O)
    b. 0.1% D_2O in (MQ H_2O)
    c. 0.5% D_2O in (MQ H_2O)
    d. 0.10% D_2O in (MQ H_2O)
    e. 1.5% D_2O in (MQ H_2O)
    f. 2.0% D_2O in (MQ H_2O)
    g. 2.5% D_2O in (MQ H_2O)
    h. 3.0% D_2O in (MQ H_2O)
    i. 3.5% D_2O in (MQ H_2O)
    j. 4.0% D_2O in (MQ H_2O)

5. Add 2µl of a 10N NaOH solution to each sample or standard

6. Add 4µl of acetone/acetonitrile solution (10µl of acetone +200µl of acetonitrile) to each sample

7. Be careful when taking samples out of the centrifuge to make sure that all of the drops are at the bottom of the tube

8. Cap and briefly centrifuge samples (~5sec) to ensure NaOH and acetonitrile react with sample

9. Let samples sit overnight (at least 10hrs)

10. Add 500µl of chloroform to samples

11. Add ~ 50mg Na_2SO_4 salt to each sample

12. Centrifuge sample for 2 minutes

13. Pipette 100µl of chloroform layer into glass insert, place inserts in GC vials and cap, then assay on a GC-MS system, EI mode (see below for parameters and references).

For doubly labelled water studies, such as total energy expenditure; TEE (1,4):

1. $H_2{}^{18}O$ standards:
    a. Blank (MQ H_2O)
    b. 0.01% $H_2{}^{18}O$ in (MQ H_2O)
    c. 0.05% $H_2{}^{18}O$ in (MQ H_2O)
    d. 0.10% $H_2{}^{18}O$ in (MQ H_2O)
    e. 0.15% $H_2{}^{18}O$ in (MQ H_2O)
    f. 0.20% $H_2{}^{18}O$ in (MQ H_2O)
    g. 0.25% $H_2{}^{18}O$ in (MQ H_2O)
    h. 0.30% $H_2{}^{18}O$ in (MQ H_2O)
    i. 0.35% $H_2{}^{18}O$ in (MQ H_2O)
    j. 0.40% $H_2{}^{18}O$ in (MQ H_2O)

2. $H_2{}^{18}O$ assay: 5 ul of blood or plasma sample or standard into a 12 x 75-mm glass tube

3. Then add ~3 mg of PCl5 to samples or standards to generate phosphoric acid; let stand for 20 min

4. To generate TMP, then react samples or standards by adding 300 µl of freshly prepared etheral-diazomethane (to derivatize samples) and allow to stand at room temperature during reaction, until the ether evaporated; may use an additional 120µl of diethyl ether in hexane solution

5. TMP is extracted by addition of 150 ul of water and 600 µl of chloroform (1:4 ratio) followed by addition of 0.5 g Na2 SO4

6. Samples are then vigorously mixed, and a small aliquot of the chloroform is transferred to a GC-MS vial and assayed on GC-MS system, EI mode

Gas Chormatography Mass Spectrometry, GC-MS (EI mode): Acetone and the TMP derivatives are analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system, and a DB-17MS capillary column (30 m x 0.25 mm x 0.25 um). The mass spectrometer is operated in the electron impact mode (EI; 70 eV), (1,4).

7. Selective ion monitoring of mass-to-charge ratios (m/z)
    a. For ^{2}H enrichments monitor acetone (58, 59) and for ^{18}O enrichments monitor TMP (140,142)

b. The ^{2}H enrichments are calculated from the $^{2}H_2O$ standard curve and the ^{18}O enrichments are calculated from the signal ratio (142)/(142 + 140).

References:

1. Gas chromatography-mass spectrometry assay of the (18) O enrichment of water as trimethyl phosphate. Brunengraber DZ, McCabe BJ, Katanik J, and Previs SF. *Anal Biochem* 306: 278–282 (2002).

2. Increased plasma membrane cholesterol in cystic fibrosis cells correlates with CFTR genotype and depends on de novo cholesterol synthesis. Fang D, West RH, Manson ME, Ruddy J, Jiang D, Previs SF, Sonawane ND, Burgess JD, Kelley TJ.Respir Res.; 11:61 (2010).

3. Triglyceride synthesis in epididymal adipose tissue: contribution of glucose and non-glucose carbon sources. Bederman IR, Foy S, Chandramouli V, Alexander JC, Previs SF. J Biol Chem.; 284(10):6101-8 (2009).

4. Novel application of the "doubly labeled" water method: measuring CO2 production and the tissue-specific dynamics of lipid and protein in vivo. Bederman IR, Dufner DA, Alexander JC, Previs SF. Am J Physiol Endocrinol Metab.; 290(5):E1048-56 (2006).