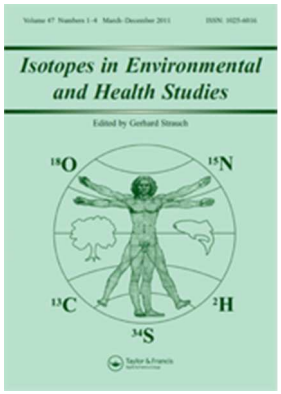

Deuterium dilution technique for body composition assessment: resolving methodological issues in children with moderate acute malnutrition

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Deuterium dilution technique for body composition assessment: resolving methodological issues in children with moderate acute malnutrition

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ABSTRACT

Childhood malnutrition is highly prevalent and associated with high mortality risk. In observational and interventional studies among malnourished children body composition is increasingly recognised as a key outcome. The deuterium dilution technique has generated high-quality data on body composition in studies of infants and young children in several settings, but its feasibility and accuracy in children suffering from moderate acute malnutrition requires further study.

Prior to a large nutritional intervention trial among children with moderate acute malnutrition we conducted pilot work to develop and adapt the deuterium dilution technique. We refined procedures for administration of isotope doses and collection of saliva. Furthermore, we established that equilibration time in local context is 3 hours.

These findings and the resulting standard operating procedures are important to improve data quality when using the deuterium dilution technique in malnutrition studies in field conditions, and may encourage a wider use of isotopes techniques.

Keywords: Stable isotope, deuterium dilution, moderate acute malnutrition, body composition.

1. Introduction

Childhood malnutrition continues to be highly prevalent and is associated with high mortality risk [1]. Traditionally, outcomes in studies addressing malnutrition have been based on anthropometry i.e. weight, weight-for-height z-score (WHZ) or mid-upper-arm circumference (MUAC). These outcomes, however, do not discriminate between fat and lean mass. Lean mass is likely to mediate many of the beneficial functional outcomes, whereas fat is important as an energy substrate, and may play a role promoting immune function, but may also increase the long term risk of chronic diseases [2].

As yet, it is unclear how lean and fat mass might benefit survival in early life. Low levels of leptin, associated with fat mass, predict mortality in children with severe-acute malnutrition (SAM) [3], but lean mass has also been proposed to promote survival [4,5]. Highly precise methods for the assessment of body composition such as air-displacement plethysmography or dual-energy X-ray absorptiometry are used in research settings, but are impractical and expensive to use in larger field studies [6,7].

The deuterium dilution technique offers a logistically more feasible alternative in field studies by obtaining measurements of total body water (TBW) from which the two components of fat and lean mass can be derived. The use of the deuterium dilution technique has generated high-quality data on body composition in studies of infants and young children in several low- and middle-income country settings (Peru [8], Gambia [9], Ethiopia [10], Nepal [11], India [12]), and in larger trials of moderate acute malnutrition (MAM) prevention in countries such as Cambodia and Kenya [13].

We chose absolute lean mass increment measured using the deuterium dilution technique as the primary outcome in a large randomized trial (Treatfood) conducted in Burkina Faso among children aged 6-23 months and suffering from MAM (weight-for-

height z score (WHZ) between –3 and –2, and/or a measure of mid-upper arm circumference (MUAC) between 115 and 124 mm) [14].

In principle, measurement of TBW by isotopic methods is relatively simple in most age groups, requiring only the collection of urine or saliva samples, before and after a single dose of deuterium labelled water. In practice, several methodological issues need to be addressed, particularly in younger age groups. We have previously evaluated different methods of administering isotope doses to healthy breastfed babies in a high-income setting, who may be unfamiliar with drinking water [15], but a wider range of methodological issues may be challenging when applying the method to children with MAM studied under difficult field conditions, such as in rural Africa.

In addition, low costs relative to isotope-ratio mass-spectrometry make Fourier transform infrared spectroscopy (FTIR) an attractive analytical option for large trials. However, FTIR requires saliva samples, but many previous isotope studies in infants and young children used urine samples, and little is known of the feasibility of saliva sampling in younger age groups, especially in challenging field conditions, such as hot and arid environments.

Prior to our trial, we therefore conducted pilot work to adapt and develop the deuterium dilution technique specifically for use in children with MAM and the local Burkinabe setting, while also testing large-scale logistical feasibility. This paper describes our efforts to (a) optimise the processes of saliva sampling and fluid administration, (b) evaluate the effects on the analysis technique of adding of sugar to the isotope dose to promote palatability, (c) minimise fractionation bias in collecting saliva, (d) minimise evaporation through isotope dose spillage, (e) evaluate precision of isotope abundance determination in aliquots taken at intervals from a single deuterium

oxide stock solution and (f) to determine isotope equilibration time in children with MAM.

2. Methods

The pilot study took place in Province du Passoré at Gonpomsom Health centre in the Northern region of Burkina Faso in January–February 2013. Characteristics of methods, participants and samples are described in the relevant sub-studies (a-f) below.

2.1 Basic protocol and analytical technique

From application of the deuterium dilution technique an estimation of TBW is obtained from which the two components of fat mass (FM) and fat free mass (FFM) can be derived. Calculation of TBW requires information on baseline level of isotopic abundance in the body water pool, the isotopic abundance of the dose solution, and post-dose levels of isotopic abundance in the body water pool.

The basic protocol for body composition assessment therefore consists of four different procedures. (1) Collection of a pre-dose saliva sample to establish the baseline level of deuterium oxide (^2H_2O) in child. (2) Administration of a dose consisting of ^2H_2O (99.8 %, Cambridge Isotope Laboratories Inc., Andover, USA), diluted with water, followed by an appropriate equilibration period. (3) Collection of a post-dose saliva sample. (4) Determination of isotope abundances in these samples by Fourier Transform Infrared Spectrometer (FTIR) (IRAffinity-1, Shimadzu, Kyoto, Japan) at St. John's Research Institute (Bangalore, India). This approach quantifies the relative concentrations of ^1H_2O versus ^2H_2O in fluid samples.

To calculate the deuterium dilution space (N) the following equation is used [16]:

$N = (T*A/a)*[(Ed-Et)/(Es-Ep)]$

Where:

T is the diluent (mass of tap water for dilution in lab + mass of dose diluted in lab) (g)

A is the mass of the dose ingested by child in field (g)

a is the mass of the dose that is further diluted in lab for FTIR analysis (g)

Ed is the isotopic abundance of the dose solution (ppm)

Et is the isotopic abundance of the tap water used to dilute the dose in lab (ppm)

Es is the isotopic abundance of the post-dose sample of saliva (ppm)

Ep is the isotopic abundance of the pre-dose sample of saliva (ppm)

Subsequently the two-component body composition can be derived as:

TBW (g) = N/1.044 (adjusted for exchange of hydrogen atoms in tissues [17,18]*)*

FFM (g) = TBW/hydration factor [19,20]

FM (g) = Body weight – FFM

In our study, the aim was not to calculate body composition using this method, but rather to optimise several discrete components of the protocol, by resolving challenges expected in children with moderate acute malnutrition. These specific issues are described below.

2.2 Pilot studies

Sub-study (a): feasibility of saliva sampling and fluid administration

The first issue concerns questions of feasibility for the field part of the basic protocol in children with MAM: how should the saliva samples be collected to obtain adequate volumes, and how should the dose be administered to get an accurately quantified measurement of dose ingested by infant. This first sub-study involved 23 children 6-30 months old, consisting of well-nourished children (n=12); MAM (n=8); and severe acute malnutrition (SAM) (n=3).

Saliva sampling

Our success criterion for saliva collection was a target volume of a minimum of 0.5 ml. In preliminary work, we tested viability of passive collection by a "cotton ball method" as recently applied in field setting, which involves putting a cotton wool ball with a string attached in the mouth of the child and wait until it is wet with saliva [13]. Subsequently active collection by lavage of the oral cavity with a "cotton stick method" was developed by applying extra layers of cotton to regular cotton sticks. Possible fractionation bias in saliva collection was tested in sub-study (c).

Fluid administration

Our success criterion for fluid (dose) administration was finding an approach that led to high acceptability whilst enabling precise measurements of the volume ingested. For ethical considerations, neither nasogastric tubes nor administration by intravenous route was considered. Oral fluid administration using water was tested using (1) disposable syringes, (2) syringes with a short butterfly catheter placed in

mouth, (3) various local cups and (4) different types of baby bottles with teat holes in various sizes. We also evaluated whether the addition (dilution) to the dose with a well-known local drink, Bissap (sugary and containing hibiscus), would improve palatability (sub-study (b)). The effect of local climatic conditions on evaporation of spillage was tested separately in sub-study (d).

Sub-study (b): the effects of adding of sugar to the dose solution

Our success criterion when evaluating the addition of Bissap to the dose solution was that analytic accuracy was maintained in the FTIR analysis. Bissap contains sugar and to our knowledge no data is available on the effect of sugar on accuracy of the $^{2}H_2O$ abundance determination by FTIR. Diluted $^{2}H_2O$ (0.1g/ml) with and without sugar (sucrose at about 2 teaspoons of sugar in 100 ml) was analysed by FTIR, to identify the possible interference of sugar.

Sub-study (c): fractionation bias in collecting saliva

This sub-study was undertaken in seven adult volunteers. The objective was to evaluate the effect on isotopic abundance of saliva sampled with an open mouth versus a closed mouth. It is unknown whether collecting saliva from an open mouth might cause isotopic fractionation, whereby heavy and light isotopes evaporate at different rates. This would alter the relative isotopic abundance of the sample and yield inaccurate data on body composition.

Ambient temperature was ~38°C [21] during the experiments. Undiluted $^{2}H_2O$, administered in proportion to body weight was ingested (0.4 g $^{2}H_2O$ per kg body weight). In the four-hour waiting period individuals minimised physical activity. No drinking or eating 30 min. before saliva sampling was permitted, nor rinsing of the mouth at any time. Saliva was collected using the cotton stick method while the

participant´s mouth was kept closed. After 3 minutes, saliva collection was repeated with an open mouth while the required volume of saliva was collected. The aim was to obtain 1 ml of saliva. If more than 0.5 ml saliva was obtained after the use of four cotton sticks the experiment was terminated. If <0.5 ml saliva additional cotton-stick sampling would be performed.

Sub-study (d): evaporation through dose spillage

When administering $^{2}H_2O$ to a child, small amounts of the dose may not be consumed, and may be spilled. If these spills are not recovered, the volume of dose administered would be over-estimated, leading eventually to an over-estimation of TBW by the same percentage magnitude. It is therefore necessary to collect spillages, for example on pre-weighed pieces of gauze (tissue), so that the mass of spillage can be quantified. In a hot, dry setting, however, these spillages may rapidly evaporate. Evaporation was tested in four small experiments with 3 pieces of gauze tested in each. Gauzes were prepared with ~1 g diluted $^{2}H_2O$, placed in sealed plastic bags and weighed with 0.01 g precision (Adam equipment: model CQT 202, United Kingdom). On opening the bags, half the pieces of gauze were curled up and pressed against the lower lip of an adult study participant to mimic "normal collection" of spillage on the face for three or seven minutes. The other half was left to "free-dry" in the air for three to seven minutes. After exposure, each piece of gauze was transferred back to its bag and reweighed.

Sub-study (e): precision of dosing isotope abundance determination from stock solution

Calculation of TBW requires precise knowledge of both the mass of the dose and the *abundance* of isotope in the dose. A Diluted Deuterium oxide Stock (DDS) is prepared by mixing commercially available $^{2}H_2O$ and water. As successive doses are removed from the stock solution, its isotopic content may change, such that the doses do not share a common isotopic abundance. The objective of this study was to test if isotope abundance of DDS samples would be similar when measured repeatedly on different days. We determined the isotopic abundance of several DDS samples (in each case sampled from the mid-bottle of a DDS solution) taken from two different DDS solutions (both ~40% solution ~400 g $^{2}H_2O$ and ~600 g H_2O). The first DDS solution was sampled on days 1,2,5,6 and 7. The second DDS solution was sampled on days 1, 2 and 5.

Sub-study (f): Equilibration time

The objective of this sub-study was to establish the $^{2}H_2O$ equilibration time in saliva for children with MAM. The experimental overview is seen in **figure 1**. Following collection of baseline saliva, 10 ml of diluted $^{2}H_2O$ (~40% concentration, ~4 g D2O) was administered as a fixed dose. Saliva samples were collected at hourly intervals (3,4 and 5 hours) in a sample of 42 children (well-nourished recovering from MAM=13; MAM n=22; SAM=7) aged 7-30 months. Since our aim was to determine equilibration time, we included in our analysis children who spilled some of the dose, because while this would negate accurate calculation of body water, it would not prevent assessment of equilibration time.

2.3 Statistics

Data were double entered in Epidata 3.1 (Odense, Denmark). Descriptive summary measures (percentage, mean +/- SEM) were used to analyze the data. No formal statistical significance testing was carried out due to limitations in sample size. The statistical software package Stata v12 (StataCorp, College station, Texas, USA) was used.

2.4 Ethics

This study was approved as part of a larger trial by the Ethics Committee for Health Research in Burkina Faso (2012-8-059) and consultative approval was obtained from the Danish National Committee on Biomedical Research Ethics (1208204).

3. Results

Sub-study (a): feasibility of saliva sampling and fluid administration

The "cotton ball method" applied in a recent trial in Cambodia, allowing a child to suck on a cotton ball with a tied-on thread hanging out the mouth hold by the investigator to prevent swallowing, failed in our setting. Children would generally not close their mouths with the cotton-ball inside and too little saliva was collected. On the other hand, the "cotton-stick method", by which we collected saliva by systematically sweeping the oral cavity, proved effective in collecting our minimum target volume of 0.5 ml.

When administrating fluid directly from a syringe to a child, initially, low acceptability and high levels of spillage were encountered. Consequently, a number of alternative techniques were investigated: a number of local cups were found to lead to excessive spillage both when applied by investigators and mothers. In healthy children

there was a tendency of better acceptance when drinking fluid from a baby bottle. But in children with MAM there was generally little will to suck from the baby bottle. Making sucking easier by increasing the size of the hole in the teat led to excessive spillage. In healthy children a well-known local drink, Bissap (sugary and containing hibiscus), was well accepted when tested as dilution. Bissap being red in colour also made it possible to discriminate between spillage from pure saliva and spillage from diluted $^{2}H_2O$. However, when tested in children with MAM, Bissap was not better accepted than water. We identified no better way to administrate fluid than by disposable syringes.

Sub-study (b): the effects of adding of sugar to the dose solution

Testing the addition of sucrose, in the laboratory setting, led to a large interfering peak which altered the appearance of the O-D stretch, and affected the calculation of $^{2}H_2O$ abundance. A known standard of 1184 ppm of $^{2}H_2O$ mixed with sucrose as a 10% solution resulted in a higher observed isotope abundance of 1560 $\pm$ 1 ppm.

Sub-study (c): fractionation bias in collecting saliva

We show the differences in the adult post dose saliva (Es) isotopic abundances collected with mouth closed and mouth open in **Table 1.** To estimate consequences for these differences in post-dose isotopic abundances relevant to our paediatric population we calculated dilution space, N, with hypothetical values for TA/a=5000, Ed-Et=1000, Ep=96. These calculated differences in dilution space were considerable, ranging from -4 to 7%.

Sub-study (d): evaporation through dose spillage

There was a 31% (SEM 3) evaporation of diluted $^{2}H_2O$ from gauzes across experimental conditions in this sub-study. Details on results of the experiments are seen in **Table 2**.

Sub-study (e): precision of dosing isotope abundance determination from stock solution

As seen in **Table 3** we found up to 8% difference in isotope abundance determination in samples from same DDS solution measured on different occasions.

Sub-study (f): Equilibration time

The children had an average weight of 7.3 kg (SEM 0.2) (range 4.81-9.8). Baseline saliva was successfully obtained in 39 of 42 children (93%) with a mean saliva volume of 1 ml (SEM 0.06). Three children failed to produce baseline saliva due to crying and dry mouths and were taken out of the study. In two children the dose was not given due to crying and these children were taken out of the study. Samples from one child were lost.

In the 36 remaining children all post-dose saliva samples were successfully collected. Equilibrium was reached at three hours as seen in **Figure 2** as abundance of deuterium in saliva (Panel A) or calculations of TBW (Panel B). In the vast majority of children there was a clear pattern. From three hours onwards, subsequent total body waters were either essentially static (defined a change of <0.1 kg TBW) or rising slightly. All of these subjects indicated equilibration of isotope by three hours. In four children a pattern was found with a rise in TBW (fall in isotope abundance) from three to four hours followed by a rise from four to five hours. We suspect these findings to be artefacts possibly being caused by a large drink between three and four hours. One child

showed three very different values, the highest value at 4 hours, and we suspect an irregularity with drinking (e.g. in the period prior to 3 hours), or saliva sampling (e.g. evaporation/fractionation at 1 or more time points) or both.

4. Discussion

Prior to a randomized trial we conducted a pilot to adapt and develop the deuterium dilution technique specifically for use in children with MAM and a hot climate and rural setting. Our findings and considerations may be of wider use for field studies in undernutrition considering body composition as an outcome.

4.1 Saliva collection

We developed a cotton-stick method that ensured a high success rate of >90% for saliva collection. Likewise, we established that isotope abundances of post-dose saliva samples were determined differently if collected with an open mouth compared to a closed mouth, possibly related to fractionation, and we recommend that saliva is collected while the mouth of the child is gently kept closed. We decided for the trial to drape the saliva collection environment with a cloth, to protect the process from direct sunlight and also to minimise risk of fractionation. At the time of our pilot the FTIR equipment used required ~0.5 ml per analysis, hence ~1 ml per duplicate isotope abundance determination. Newer FTIR systems allow duplicate analysis of as little as 60 microliters which will ease the collection of sample material for future trials. To minimise risk of contamination of saliva samples, we physically separated the location for dosing and saliva collection. In addition, a 30 minutes fasting period saliva before sampling was requested including breastfeeding.

4.2 Dose and administration

When running a large trial, it is important to simulate what the highest and lowest isotope abundance would be from putting the same dose into a small and a large child. If the child was very big, the post-dose enrichment would be low; if the child was small, the dose would be high. It depends what range of isotope abundances the FTIR lab are able to cover.

We used diluted $^{2}H_2O$ for dosing, as undiluted $^{2}H_2O$ may lead to inaccuracy of estimation of dose ingested by child. $^{2}H_2O$ is enriched relative to water vapor in the air and will fractionate rapidly**.** The risk is that not all the dose swallowed and evaporates off the surface of the mouth and throat**.** With a dose of 5 g isotope, a loss of 0.05 g in this way will result in a 1% dosing error, i.e. a 1% body water error. In the same way, scales weighing accurate to at least 0.01 g are necessary to minimize error on the calculated mass of dose administered.

By diluting the dose with potable water, the relative error of evaporation will be reduced. The magnitude of dilution of the dose is influenced by both biological and practical logistical considerations. To cover all weight ranges in our trial and to make logistics as easy as possible for the trial, all children received 5 g $^{2}H_2O$ in about 10 mL DDS, meaning that the total dose to be consumed was ~10 mL.

When running a large trial it is tempting to reduce the workload by determining isotope abundance in the DDS solutions and use this value for all children having received an aliquot from this solution. However, in this pilot we established substantial variability in samples from same DDS aliquoted on different time points. Subsequently, we decided for our main trial to analyse isotopic abundance in every individual dose given a so-called individual study dose (ISD).

We tested various techniques for dosing, and ended up with the conventional use of disposables syringes. Likewise, we had to abandon the idea of adding a local flavour

to the dose as the sugar content in the local drink identified would have interfered with isotope abundance analysis in FTIR analysis. For studies analysing samples on mass spectrometry the addition of sugar would still be an option. However, from a logistical point of view using disposable material and plain water as diluent makes a large study more feasible. We established that in the local setting evaporation takes place extremely fast with the risk of overestimation of dose in child. For our trial we made procedures to minimize time dose was exposed to environment and to instantly place gauze with spillage into airtight containers.

We established that equilibration time in saliva in local context is 3 hours, just a few children diverted from this pattern which we consider was probably related to the ingestion of large drinks during the equilibration period. Previous work in younger age groups has been conducted on healthy subjects in high income settings. Most work on infants has used a "back extrapolations" method *[16]* to calculate body water. This uses repeat post-dose urine samples across several days, since body water was typically calculated as part of the doubly labelled water method for estimation of energy expenditure. Other studies of infants, children and adults suggest that isotopic equilibration typically occurs in saliva within three hours *[22,23]*, however a longer period may be required for those overweight. No information is available on the time required for equilibration in undernourished children.

Furthermore, as some children during dosing would spit in a manner or degree that could not be collected by gauze, we decided for our trial to include estimation of dose loss if administration was not perfect. Also to avoid the risk of children vomiting after the dosing we imposed an observation period following dose administration.

During our trial we improved the psycho-social environment by using only experienced local staff and creating a playful/calm atmosphere.

The experiences from our pilot study let to development of standard operational procedures for management of the deuterium dilution techniques in our trial that can be found as online supplemental material. *Online supplemental material 1:* Laboratory Deuterium oxide dilution, Sampling and Aliquoting. *Online supplemental material 2:* Dose administration in field and *Online supplemental material 3*: Field data collection sheet.

A limitation of our study is that we did not propagate our raw isotopic data to body water values and hence body composition, which would potentially have allowed us to assess the overall accuracy of the deuterium technique relative to a reference body composition method (eg air-displacement plethysmography). We did not have access to any such reference method in this field setting. Such a validation study would be a valuable future aim. Nevertheless our work reported here has clarified how to optimise the protocol for collecting isotopic data in children with moderate acute malnutrition.

5. Conclusion

We established how to collect saliva and dose diluted $^{2}H_2O$ in a local Burkinabe setting prior to a large intervention study in children with MAM. Our pilot findings and standard operating procedures developed for the main trial may be of wider use for groups considering using the deuterium dilution technique in malnutrition studies in field conditions. However, we encourage that a local pilot is always undertaken prior to the application of this method.

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Table 1: The effect of closed vs open mouth on post-dose saliva enrichment in 7 adults

ID	Es1	Es2	Δ Es	Es1-Ep	Es2-Ep	N1	N2	ΔN	%ΔN
1	583	572	-11	487	476	10276	10508	232	2.3
2	586	581	-5	490	485	10200	10305	105	1.0
3	652	615	-38	556	519	8987	9641	655	7.3
4	738	745	7	642	649	7792	7709	-83	-1.1
5	555	574	19	459	478	10897	10453	-443	-4.1
6	802	796	-6	706	700	7080	7143	63	0.9
7	730	724	-7	634	628	7880	7967	87	1.1

Es1 is isotope abundance of saliva collected with mouth closed. Es2 is isotope abundance of saliva collected with open mouth. To calculate dilution space N hypothetical values were used for TA/a=5000, Ed-Et=1000, Ep=96 (not shown in table)

Table 2: Effect of evaporation of spillage

Experiments	%Δ weight (SEM)
a. Gauze touching skin, 3 min	25 (8)
b. Gauze touching skin, 7 min	36 (3)
c. Loose gauze, 3 min	22 (2)
d. Loose gauze, 7 min	39 (3)

Percentage weight loss of ~ 1 g of diluted $^{2}H_2O$. Determined in triplicate measurements in each of a-d

Table 3: Accuracy in dosing abundance determination from diluted deuterium oxide stock solution

DDS #	Day #	A	twdil(g)	a (g)	Ed	Es-Ep	TA/a	N	%ΔN
1	1	10	49.9	0.106	816.3	1000	4699	5756.3	-
1	2	10	49.4	0.101	794.5	1000	4903	6171.3	7.2
1	5	10	49.5	0.103	820.6	1000	4825	5880.2	2.2
1	6	10	49.2	0.100	793.9	1000	4922	6200.0	7.7
1	7	10	49.2	0.104	829.3	1000	4754	5732.8	-0.4
2	1	10	49.1	0.100	799.7	1000	4906	6135.4	-
2	2	10	49.3	0.102	804.9	1000	4834	6004.8	-2.1
2	5	10	48.9	0.103	820.7	1000	4771	5813.7	-5.2

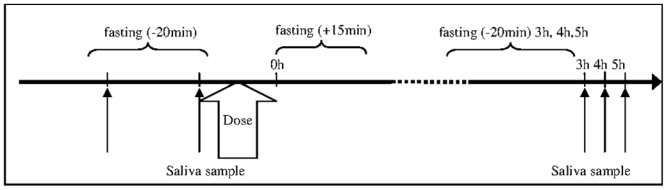
DDS# indicates which of two diluted deuterium oxide stocks that was sampled. Day indicates day of sampling. Twdil is the mass of tap water used to analyse the DDS while a is the mass of DDS used to analyse isotope abundance. Ed is the determined dose abundance, while A, Es-Ep, TA/a are hypothetical values to calculate the dilution space in a child similar to our study population.

Figure 1

Experimental overview to establish equilibration time sub-study (f)

Figure 2

Panel A: Deuterium oxide abundance of post dose saliva. Every line indicates a child that has had isotope abundance in saliva determined at 3, 4 and 5 hrs post dosage. Panel B: Calculated total body waters.



Experimental overview to establish equilibration time sub-study (f)
Experimental overview to estab
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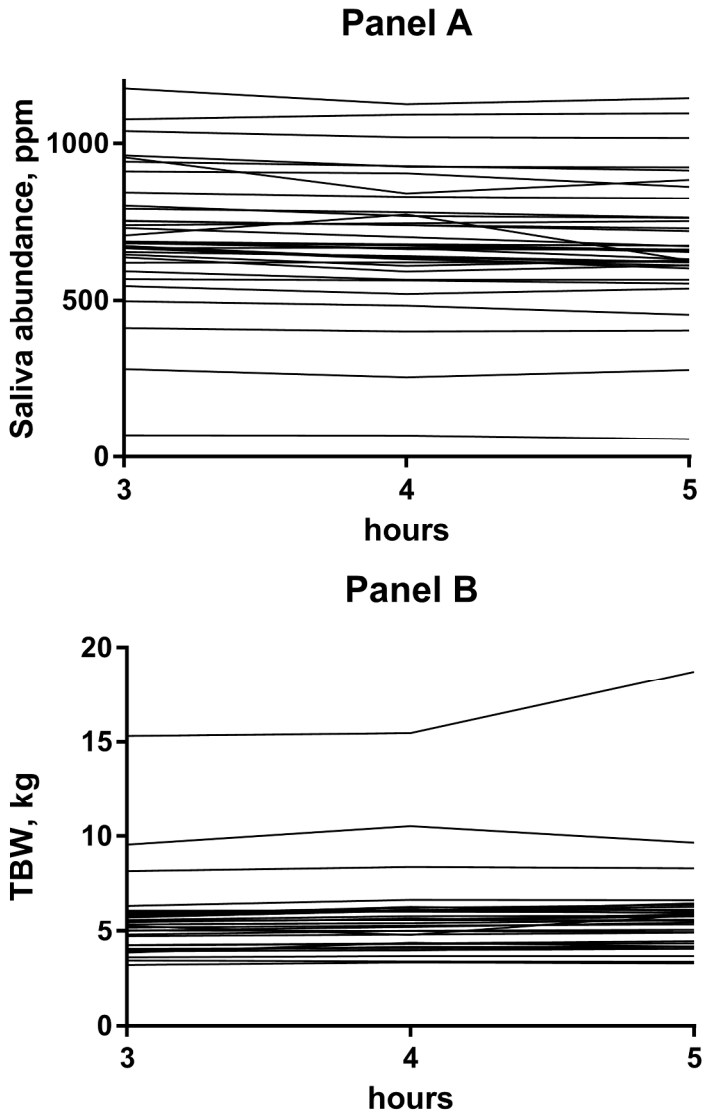


Figure 2
Panel A: Deuterium oxide abundance of post dose saliva. Every line indicates a child that has had abundance in saliva determined at 3, 4 and 5 hrs post dosage. Panel B: Calculated total body waters.

189x271mm (300 x 300 DPI)

ONLINE SUPPLEMENTAL MATERIAL 1

Laboratory Deuterium oxide dilution, Sampling and Aliquoting

Preparation of DDS-batch

A Diluted Deuterium oxide Stock (DDS) is prepared by mixing bottled-water (brand: Lafi) and 99.8% D2O (brand: Cambridge Isotope). Mix DDS in a clean 1 L borosilicate bottle with a PTFE facing disc screw cap (mixing–bottle). Weigh on an electronic scale (range: 0-2 kg (0.1 g)). Keep lids on bottles during weighing to diminish evaporation.

a) Calibrate weight. Check against a standard object.
b) Tare bottle + lid (to 0.1 g).
c) Dispense ~ 450 mL 99.8% D2O to mixing-bottle using a glass cylinder.
d) Weigh D2O in the mixing-bottle (~ 500 g) and record exact weight in the DDS-log
e) Add ~ 500 mL of bottled-water to the mixing bottle using a glass cylinder.
f) Weigh what is now a new DDS (~ 1000 g) and record exact weight in the DDS-log.
g) Mix DDS thoroughly by systematically turning mixing-bottle upside down 15 times before sampling and aliquoting.

Every new DDS is assigned a unique DDS-code: *T-DDS-##*. Where ## is a new consecutive number.

a) When dispensing with new pipette tip DDS must be drawn back and forth 5 times to saturate the plastic pipette tip with D2O.
b) For every new DDS collect a ~2 ml sample (from the middle of the mixing-bottle) in a cryotube with O-ring. Label with *T-DDS-##* and store at -20°.

Data to register in the physical DDS-log book and electronically	**Date**	***T-DDS-##***	**Weight D2O**	**Weight DDS**

Preparation of Dose-Bottles

12 ml DDS is out-portioned in a Dose-Bottle (10 ml = Individual Study Dose (ISD) will be administered to study participant; the additional 2 ml = ISD-sample will establish the exact abundance of D2O in Dose-Bottle). One DDS is enough for ~ 78 Dose-bottles.

c) Dispense exactly 12 ml DDS by using a pipette into a Dose-Bottle that is immediately closed.
d) Add label on the Dose-Bottle with: *T-DDS-##*.
e) After Aliqouting 10 Dose-Bottles mix remaining DDS thoroughly by systematically turning mixing-bottle upside down 15 times.
f) Place Dose-Bottle in fridge (4°C) in until field usage.

Logistics

DDS is mixed and Dose-bottles prepared according to field needs and kept in fridge (4°C). 1 DDS contains a volume for ~ 78 Dose-Bottles (if field needs are small freeze half the Dose-Bottles). Every morning Dose-bottles are transported from lab to field-sites in a cold bag. Unused Dose-bottles are returned to fridge in lab every evening and a dot is added to the label indicating a "day-in-the-field". Dose-Bottle with most dots should be used first in field. However, Dose-Bottles with five dots must be thrown out.

Background for choosing 5 g D20:
In TreatFood children aged 6-23 months with MAM are included. Intervention is for 3 months. An ISD is administered at t=0 and t=3 months. Gross Estimation of weight range: 5.1-12.5 kg (6 months-old- girls **-3SD**/ 26 months-old-boy **median** (based on weight-for-height WHO standards)).
The proportion of water in the body varies in relation to fatness. In thin children, lean mass can be >90% of the weight and if hydration of lean tissue were 78%, around 70% of weight could be water - possibly even more as malnutrition can lead to water retention. With 5 g of D20 and an hydration of body mass of 70 % the estimated range of enrichment according to weight range will be 5 g D20/(weight *.7)= ~570-1400 ppm.

Background for DDS mixing D20 dilution:
Calculations made on the assumption that temperature is 25 C°
Density D2O: 1.105 g/mL
Density H2O: 0.997 g/mL

Characteristics of one **DDS:**
Volume: 950 ml (450 ml D2O + 500 ml H2O)
Weight: 995 gr (497 gr D2O + 498 gr H2O)
Weight proportion D2O/DDS ~ 0,5
1 mL of DDS weighs: ~ 1.048 gr
1 mL of DDS contains : ~ 0.523 gr D2O (1.048 gr * 0.5**)**

5 gr D2O in: ~ 10 mL DDS or Precisely 9.56 ml DDS (5 gr D2O/0.523 gr D2O/ml DDS)

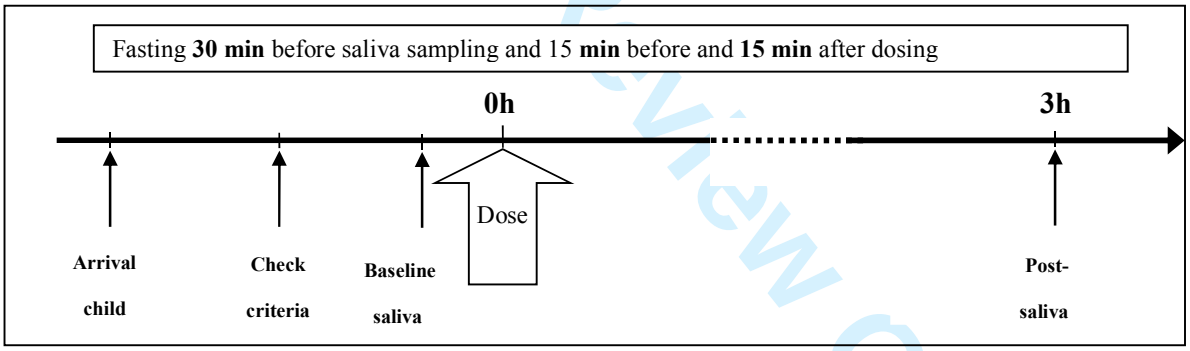
ONLINE SUPPLEMENTAL MATERIAL 2

Dose administration in field

A successful dose administration is: When the **precise** weight of D2O swallowed by child is known and when 0.5-1 ml of saliva has been collected before and after dosing.

The method takes time, precision and patience! Procedures must never be rushed and a child-friendly environment must at all times be maintained. Activities are separated in three different locations in field: Observation (OBS), Saliva sampling (SAL) and dosing (DOSE). All information must be meticulously recorded according to the Deuterium Data Collection Sheet of individual children and in a general Deuterium Field Logbook. The Electronic balance is calibrated every morning and precision tested with an object of known weight.

Overview:



1) **Checking criteria and observing fasting before baseline saliva sampling (OBS)**
   a) Check criteria: child must have passed registration and anthropometry.
   b) Observe that child is fasting 30 minutes prior to saliva sampling.
   c) Make sure Deuterium Data Collection Sheet is correctly filled in.

2) **Baseline saliva sampling (SAL)**
   a) Confirm that child has been fasting 30 min prior to baseline saliva sampling.
   b) Mark (with pen) a 2 ml cryotube with o-ring and red cap with study-ID of child: T####.
   c) Explain procedure to caretaker.
   d) Wipe the mouth of the child with dispensable paper tissue before start.

e) Wear new dry gloves before collection. Any kind of fluid on fingers can contaminate results.

f) Collect saliva out of bright sunlight with an already prepared cotton-stick[1]. Systematically collect saliva with cotton stick with one hand while the other hand gently closes the mouth of the child. Two persons might be needed.

g) When first cotton stick is introduced in child´s mouth start clock to time collection time.

h) Remove the cotton now soaked with saliva from cotton stick and extract saliva using the plunger from a new 10 ml syringe into cryotube. **Close the lid well immediately**.

i) It is often necessary to repeat collection procedure a number of times to obtain sufficient saliva.

j) Target volume is 1 ml. Absolute minimum volume is 0,5 ml.

k) As long as volume of 0.5 ml saliva has not been reached collection must be continued. If 0.5 ml saliva or more has been collected after 10 minutes collection is discontinued. (NB: it should be a very rare situation if collection of saliva must be given up before reaching 0.5 ml)

l) The same 10 ml syringe and plunger can be used for same saliva sample. If syringe is polluted a new syringe is needed. While collecting saliva the syringe is placed on a clean paper tissue. Paper tissues are changed if wet and always between children.

m) Label cryotube with pre-printed labels: At inclusion T####-S-ZERO-IN and at 3 months T####-S-ZERO-EX.

n) Immediately place baseline saliva sample in small zip locked plastic bag placed in "low abundance" cold bag (4ºC). All small bags of baseline saliva sampling are collected in a big zip locked bag to separate baseline from post dose saliva samples.

o) Discard cotton-sticks, syringe and gloves.

p) Make sure Deuterium Data Collection Sheet is correctly filled in.

3) Observe fasting before dosing (OBS)

a) Child must be confirmed fasting for min. 15 min prior to dosing

b) Make sure Deuterium Data Collection Sheet is correctly filled in.

4) Deuterium dosing (DOSE)

a) Carefully note all results continuously as indicated on the *Deuterium data collection form.*

b) Confirm that child has been fasting 15 min. prior to dose administration.

c) Label a 2 ml cryotube with o-ring and yellow cap: T####-ISD-IN and at 3 months T####-ISD-EX.

d) Explain caretaker procedure and allow time to play and interact with child.

[1] Cotton sticks are prepared with an extra layer of cotton. Cotton sticks must be prepared wearing new gloves and away from the daily activities in the hangar to avoid contamination with D2O.

e) Wear new gloves. Get Dose-Bottles with DDS from cold bag. Always first use Dose-Bottles with most "field-days" indicated on label.

f) Mix by turning Dose-Bottle upside-down systematically 15 times.

g) Draw 10 ml of DDS into a new 10 ml syringe using a 19G needle. The content in the syringe is now an individual study dose (ISD). If syringe is not completely dry on outside wipe with paper tissue that is immediately disposed.

h) Place the full syringe in a sealed plastic bag together with 5 new pieces of gauze. Make sure no fluid is squeezed out of syringe while handling.

i) With a single use plastic pipette dispense the remaining approx. 2 mL from the Dose-Bottle into a labelled cryo-tube. Dispose the pipette after use.

j) All ISD samples from same day are placed in a small zip plastic bag. ISD samples have a high concentration of D2O and must never be stored with saliva samples. ISD sample are placed in "high abundance" cold bag (4ºC) also used for Dose-Bottles.

k) Place plastic container on scale and Tare scale. Place plastic bag with content in plastic container. Make sure that plastic bag is completely inside the plastic container. Weigh plastic bag with content with two decimals precision.

l) Dry the infant around the mouth with a paper tissue before dosing is initiated. Dispose tissue.

m) Be prepared for collecting any ISD spillage during administration on the pre-weighed tissues (gauzes). Pure drooling to be collected on disposable paper tissue.

n) Administer ISD slowly and precisely in the corner of the mouth. Collect ISD spillage on gauze. Wet gauze is placed in plastic bag and replaced with a dry piece of gauze. Plastic bag is always kept closed. 2 persons will often be needed for the procedure.

o) If 5 pieces of gauze is not sufficient extra gauze (that has been pre-weighed) are used.

p) After administration tare scale with plastic container. Place plastic bag with all content previously being weighed (ISD syringe + 5 pieces of gauze) in container and re-weigh. Note result on *data collection form*. If extra gauze has been used the procedure is repeated here. The difference in weight of bag with content before and after administration = weight of dose in child.

q) Note the exact time of end of dosing in *data collection form.*

r) Discard gloves and all material used.

5) Post dosing observation (OBS)

a) The child must be observed 15 min post dose fasting. If the child is regurgitating in this time period it should be noted in the *data collection form.*

6) Observing fasting before post dose saliva sampling (OBS)

a) The child must be observed fasting 30 min. before post dose saliva sample.

b) Make sure Deuterium Data Collection Sheet is correctly filled in.

7) Post dose saliva sampling (SAL)

a) Starting exactly 3 hours after completion of D2O dosing the post dose saliva sample is collected following exactly the same steps as for baseline saliva collection. Use cryotube with o-ring and green cap. Label Post dose saliva sample T####-S-POST-IN and at 3 months T####-S-POST-EX

b) Immediately place post dose saliva sample in small zip locked plastic bag placed in "low abundance" cold bag (4ºC). All small bags of post dose saliva sampling are collected in a big zip locked bag to separate baseline from post dose saliva samples.

8) **Central Lab**

a) At the end of the day all samples are transported to central lab in cold bags and as fast as possible frozen (-20ºC) until analysis to minimise bacterial growth

9) **Cotton stick preparation**

a) Cotton sticks are prepared in the morning in the saliva room.

b) 20 cotton sticks are prepared in a new plastic bag. More bags can be prepared at the time.

c) Once removed cotton sticks must not be reintroduced in plastic bag.

Field equipment needed

In field: Saliva sampling	**In field: Administration/sampling of D2O**
Cotton sticks with extra cotton	Electronic balance (precision 0.01)
10 ml syringes	10 mL syringes
Disposable paper tissues	Zip-lock plastic bag
2 ml cryotubes with labels	19 G needle
Gloves	Safety box for disposed needles
Zip-lock plastic bag	2 ml cryotubes with labels
Stop watch	Disposable paper tissues
Cold-chain	Gaze-tissue approx. 7.5 X 7.5 cm (non-sterile)
	Dose-bottles

ONLINE SUPPLEMENTAL MATERIAL 3

ZERO SALIVA: **A1**.Fasting 30 min. before saliva collection? ☐ 0. **No** ☐ **1. Yes**

A2. Collection time from first cotton-stick in mouth to full cryotube : _____min.

A3. Number of Cotton-balls extracted?_____ **A4.** How much saliva was collected:_____ml

DEUTERIUM: **B1.** Fasting 15 min. before dosing? **0. No** ☐ **1. Yes** ☐

B2. DDS-#_____ **B3.** Time when starting dosing _____(hh/mm)

B4. WEIGHT **BEFORE** (weight of kit before dosing)_____gr.(XX.XX)

B5. WEIGHT **AFTER** (weight of kit after dosing)_____gr.(XX.XX)

B6. If extra tissues weight before:_____g **B7.** If extra tissues weight after:_____g

B8. Was full dose administered to child? ☐ **0. No** ☐ **1. Yes**

B9. Was there spillage on gauze? ☐ **0. No** ☐ **1. Yes**

B10. Spillage outside gauze?

☐ **None** ☐ **1-3 drops** ☐ **> 3 drops - 0.5 ml** ☐ **> 0.5 -1 ml** ☐ **>1ml**

B11. Comments for administration if any (max 80 charaters)

B12. **Dose administered by full name + ID number :**_____

B13. Time when finishing dosing:____ (hh/mm) **B14** collect post saliva at _____(hh/mm)

B15. Did child regurgitate within 15 minutes of receiving D20? ☐ **0. No** ☐ **1. Yes**

B16. Has child been observed fasting 15 min after saliva collection? ☐ **0. No** ☐ **1. Yes**

POST SALIVA: **C1**.Child fasting 30 min. before saliva collection? ☐ **0. No** ☐ **1. Yes**

C2. Collection time from first cotton-ball in mouth till cryotube full : _____min.

C3. Number of Cotton-balls extracted?_____ **C4.** How much saliva was collected:_____ml

C5. Time when finishing saliva collection:____ (hh/mm)

B6. Comments for saliva collection zero and post-dose any (max 80 charaters)