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DEUTERIUM DILUTION METHOD FOR DETERMINING THE BREAST MILK INTAKE OF BABIES

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CONTENTS

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Abstract

Various methods for measuring the milk intake of breast fed babies are available. Most methods are time consuming, cumbersome and inaccurate. This report describes various methods used for Breast Milk intake measurement with emphasis on the D_2O dilution method. The methodology of the D_2O dilution method is now streamlined at RIAD for measuring the milk intake of babies. Advantages and disadvantages of all the methods used to determine the breast milk intake of the infants are also included in the present report. For the D_2O dilution method, an oral administration of small dose of deuterium oxide (^2H_2O) is given to the mothers after collecting pre-dose samples. The post dose samples of urine from the baby and saliva from the mother are collected at 24 hours intervals over a 14 days period. These samples are analyzed on isotope ratio Mass spectrometer. The D/H values obtained over a 14 days period are then extrapolated to calculate the milk intake of babies using the Microsoft Excel Computer programme designed for this purpose.

1. INTRODUCTION

Measurement of breast milk intake of babies is important both at national and international levels. Internationally, accurate estimates are important for the of estimates of nutrient requirements such as those suggested by the FAO / WHO / UNU expert consultation [1]. Nationally, accurate estimates of breast milk intake are an essential component for policy makers and planners of maternal and child health in the country. This is particularly important in Pakistan where the introduction of solid food is late and have low content of energy and protein. Nutrition and growth studies rely on accurate measurements of breast milk intake that are practical and interfere minimally with nursing. Three conventional methods generally used for the measurement of breast milk intake are test weighing, flow meter method and tracer technique.

2. MILK OUTPUT MEASUREMENT TECHNIQUES

2.1 Test weighing method

The traditional way of measuring milk-intake of the babies is test weighing. In this method, the weight of milk consumed by the infant is estimated as the difference between the weights taken before and after each feed. The daily intake is the sum of all individual feeds during the day [2]. Several modifications of the test weighing procedure have been used in an effort to accommodate more frequent feeding. These include milk expression, restricting access of the child to the breast (i.e. imposition of a schedule) and test weighing four time per day irrespective of feeds, with measurement of urinary and fecal losses [2].

2.2 Flow meter method

A thin plastic shield is placed over the nipple, and the flow of milk through an artificial teat is measured using a Doppler ultrasound technique. This permits detailed studies during individual feeds and, if a small sampling line is also

introduced, it is possible to achieve simultaneous estimates of the composition and the flow rate as the child feeds. There is obvious a great potential for such a device in a hospital or metabolic ward, but for community studies the technological complications involved in this method might reduce its use [3].

2.3 Tracer technique

The limitations in the test weighing and flow meter method have led to the development of a procedure based on the measurement of the water turn over rate of the body [4] by using a tracer. Initially, tritium (^3H) was used as a tracer for the measurement of water turn over rates and it had been widely used in the animals.

In 1977, Halliday and Miller developed a technique for the precise measurement of the Total Body Water using the deuterium oxide (D_2O) [5]. The radioactive or Tritium method evolved for animal studies was then modified to be used in humans by Coward et.al., [6] at Dunn Nutrition Laboratories in 1979, when the stable isotope of hydrogen, deuterium (^2H) was introduced as a tracer. Now, the tracer methods for measuring milk intakes have been widely used in many species including human beings [7-12]. Tracer method can be divided into two on the basis of the choice of isotope used.

a) Radioactive method

b) Non radioactive method (stable isotope method)

2.3.1 *Radioactive Method*

In radioactive method Tritium (^3H) is used as a tracer. Scintillation counter is used for the measurement of radioactivity of samples. This method involves radiation to the subject, making the technique unsuitable for children and women of child bearing age [9,10,11] or when repeated measurement over a short period are necessary.

2.3.2 *Non radioactive or stable isotope method*

The basic principle of stable isotope method is the same as of tritium tracer method, except Deuterium is used to measure the water turn over rates. which are then extrapolated to calculate milk intake or out put. The samples are analyzed on the mass spectrometer [13]. Stable isotopes are preferable in studies involving human subjects, especially pregnant women and children [14]. This method is non radioactive and non-invasive. Since this method involves the dilution of the dose of deuterium either in the mother or the baby, it is therefore referred to as deuterium dilution method.

3. D_2O DILUTION METHOD

D_2O dilution method has been commonly used in developed and developing countries for measuring the breast milk intake of the babies [6, 15]. The dose of deuterium oxide (D_2O) can be given to the mother or to the baby (14). In dose-to-baby method, measurements of milk intake in breast fed babies are done by giving D_2O to the babies and milk intake is calculated from the exponential slopes of tracer disappreance curve [6]. This method mostly used by zoologist in the field studies but its use is limited due to some drawbacks. The most likely error is an over estimate occurring as a result of counting water entry across skin and lungs in humid atmosphere and unmonitored oral fluid intakes, as breast milk intake.

These problems are avoided if oral dose of D_2O is given to the mother. The D_2O gradually mixes in the water pool of mother, When she feeds the baby, the D_2O is also transferred into the baby. Samples of any body fluid (Saliva, breast Milk, urine etc) of the mother and baby are collected over a 14 day period and analyzed on mass spectrometer for D/H ratio. An enrichment curve of the baby and declining curve of D/H for the mother is obtained and extrapolated for the calculation of breast milk intake of the baby [Fig. 1&2].

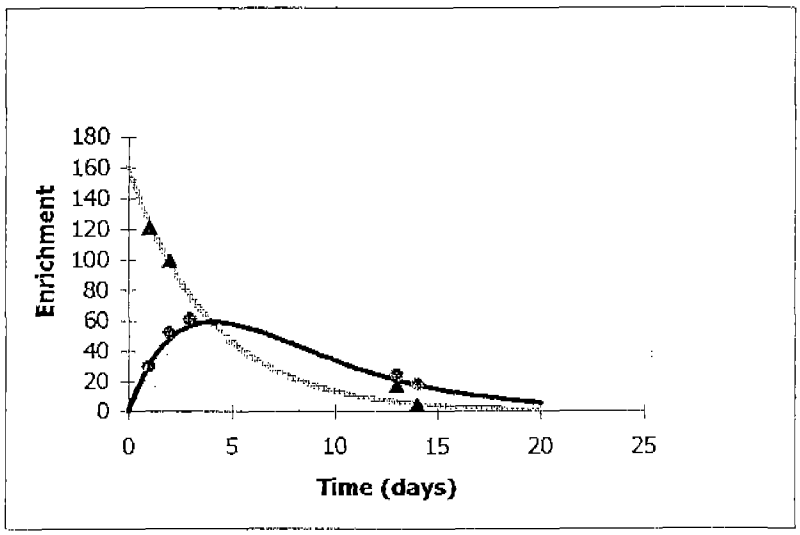

Fig.1 Typical curves showing the declining curve of ^2H concentration in saliva of (▲) mother and enrichment curve in the urine of infant (•).

3.1 Principle

The D_2O method involves enriching the body water of the subject with an isotope of hydrogen (^2H) and then determining the wash out kinetics as the concentration decline exponentially toward natural abundance levels (Fig. 2).

D_2O is used as a tracer to follow the movement of water (tracer) into and out of the body water pools of mother and her breast fed baby. The subject's total body water is seen as a single compartment and newly introduced tracer is considered to be rapidly and uniformly distributed . Following are two main assumptions of the method:

1) The deuterium is mixed thoroughly and evenly in the body water.

2) The pool size remains constant during the study.

The concentration of hydrogen isotope, nearly all of which remains associated with water molecules, decreases as a result of dilution of body water by new unlabelled water (consumed as food and drink and produced during oxidation of foodstuffs), coupled with the simultaneous loss of labelled water via

evaporation from lungs and skin, and via excretion and secretion. The rate constant for ^2H is derived as the slope of log ^2H enrichment against time (Fig. 2) and is a measure of the rate of water movement through the subject [14].

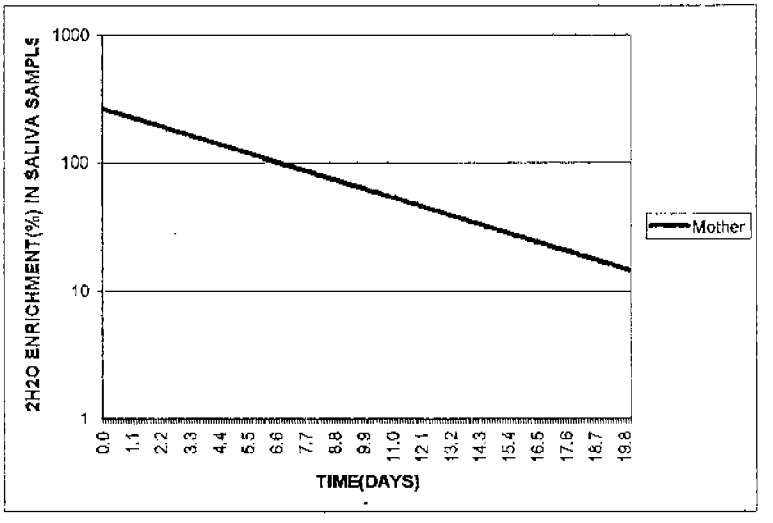

Fig. 2 D/H enrichment in saliva samples of mother after an oral dose of D_2O. (Log curve).

3.2 Kinetics

In dose-to-the-mother method, the mother baby pair is modeled with respect to movement of water through the two pool system (one is for the mother and another is for the baby). A dose of isotope D_2O(heavy water) given to the mother is viewed as mixing with the mother's body pool. It will be washed out of the mother by incoming naturally abundant water and some of it also transferred to the baby, to be lost from the system as a consequence of declining enrichment in the mother and additional water inputs in the baby, if the latter is being supplemented. A diagram illustrating this model is shown in Figure 3. Water flow is indicated by F and the subscripts (b, m and o stand for baby, mother and out side) indicate the direction. Thus Fmo is to the mother from the out side. Fom

and Fbm are the flow from the mother to outside & to baby respectively. V_m and V_b are the total body water of mother and baby respectively [14].

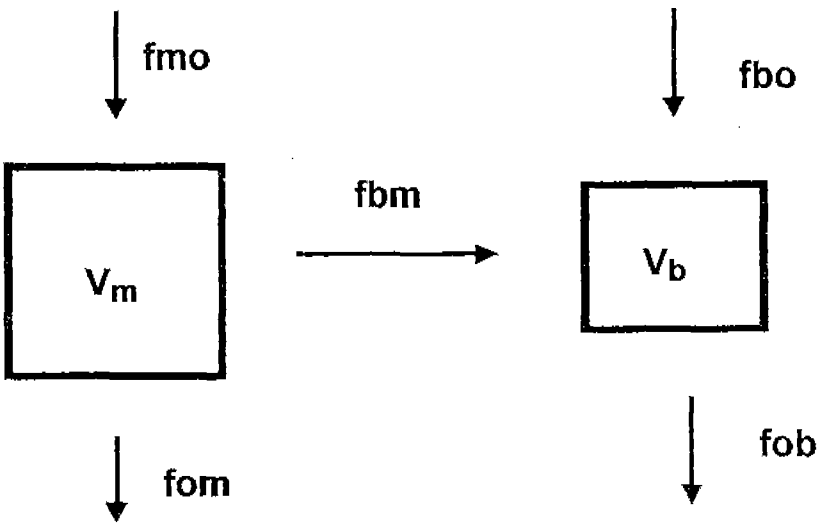

Fig. 3 Model for dose-to-mother method. V indicates body water volumes for the mother (Vm) and her baby (Vb) and f defines water flux rates. Subscripts show the direction of flow: e.g. fbm is flow to the baby from the mother and fom to the outside from the mother.

Ref : Coward WA (1981), Breast milk intake measurement in mixed fed infants by administration of deuterium oxide to their mothers, 36C, 141-148.

3.3 Sampling and storage

In dose-to-mother method, the base line samples of Saliva/ breast milk of the mother and urine of the babies is collected after which mother ingests 70 g dose of Deuterium Oxide. The post dose samples of saliva/breast milk from mother and Urine from the baby is collected on day 1,2,3,4,5,6 and 13&14. The saliva samples are collected either through cotton wool placed in the mouth of the mothers or by direct collection from the subject into collection vial. The urine samples from the baby can be collected either through cotton wool placed in the

diaper or collection into pot and then transferred into the bottles. The collected samples are stored at –15°C in a deep freezer until analyzed.

3.4 Apparatus

Reduction Vessels: The reduction vessel with one way high vacuum, 9-mm bore stopcock (right angle) called Kontess tubes were fabricated at PINSTECH glass blowing workshop using procured glass ware (Fig. 4).

Heating Block: The heating block consisted of aluminium block with 6 holes. The temperature range can be controlled between 100 to 500°C. This heating block was fabricated locally (Fig. 5).

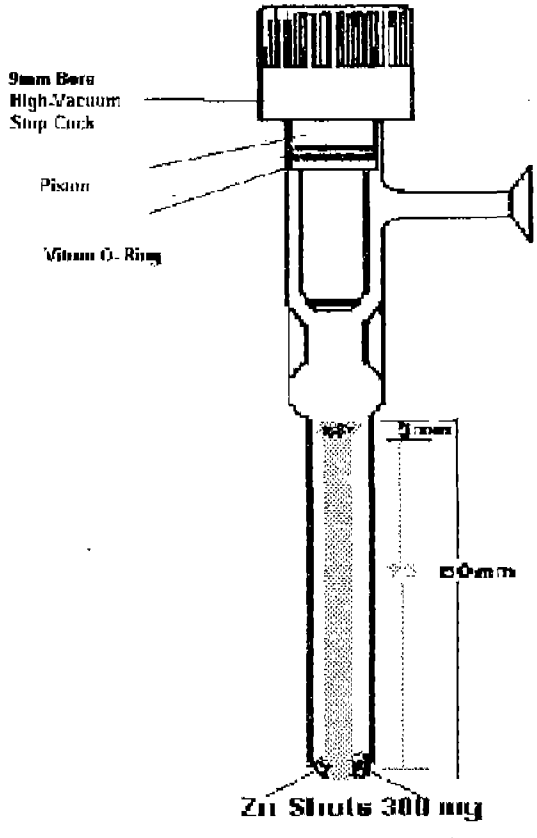

Fig. 4 Pyrex Reduction Vessel (Kontess Ampoule)

Zinc Shots (AnalaR, BDH): The Zinc shots were sieved into coarse and fine granules with a steel sieve. After washing with water the Zinc Shots were washed with 1.0% HNO_3 to remove oxides from the surface and subsequently stirred for 1 min and rinsed with deionized water. The zinc shots were again washed with acetone to remove water and degassed under vacuum and heated at 250°C for 2 hours. To avoid from oxidation by atmospheric oxygen, the clean zinc shots are stored in ampoules and attached with a vacuum pump until use.

Cup: Cups of about 8cm (length) are used. The cup method is more frequently used in the biological samples like saliva, milk, urine, sweat etc (Fig. 4).

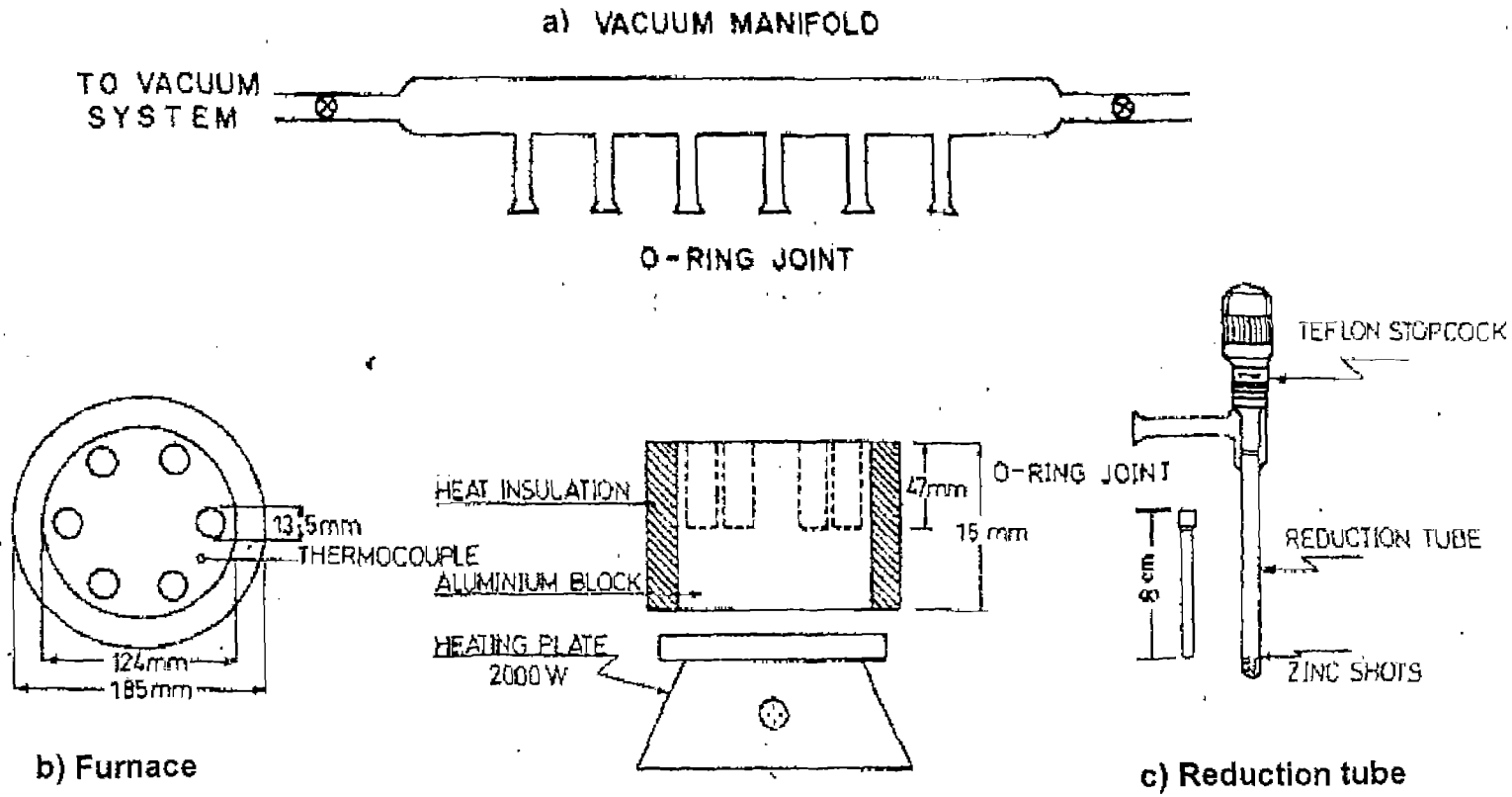

Fig. 5 A Preparation set up: (a) Vacuum manifold with provision of six tubes. (b) Furnace with six holes & (c) Reduction vessel.

Preparation of samples for D/H from the biological fluids (Zn Reduction Method)

Zinc reduction technique, originally reported by Kendall and Coplen [16] for water sample preparation is useful for preparation of biological samples for D/H determination [17]. However, other methods like equilibration method using platinum on aluminium catalyst or Hokko beads can also be employed [18]. In Zn-shots reduction method, the water (in biological fluid) is reduced using Zn shot for the production of H_2 gas. The gas is then analyzed on mass spectrometer for δ determination. Zn shot method requires only small amount of biological fluids (eight micro liters). The reaction is as follows:

$$H_2O + Zn \longrightarrow ZnO + H_2$$

Procedure:

1) The frozen samples are thawed at room temperature. Breast Milk samples require centrifugation for 10 minutes at 15,000 rpm. The fat layer at top is removed and liquid beneath is transferred into storage bottle. Watery portion is used for analysis. Saliva samples are also centrifuged to remove solid particles and supernatant used for processing.

2) 300 mg of medium zinc shots (1 mm diameter) are loaded into a reduction vessel, the vessel is attached to the vacuum line and evacuated first with the rotary vacuum pump and then with the diffusion pump ($<10^{-3}$ mbar). The vacuum line can have a provision of loading several ampoules at the same time.

3) Zn shots in the loaded ampoules are heated with blower for 15-20 seconds to remove moisture and allow to cool for approximately 12 minutes.

4) Argon gas is filled in the line and then in the ampoules.

5) 8ul of samples is introduced into the cup with a micropipette. The cup is than placed in the numbered Kontess ampoule with the help of forceps. The stopcock are replaced immediately.

6) Samples in the cup are frozen using liquid nitrogen for six minutes and evacuated to remove the argon gas (2 minutes with rotary vacuum pump and 1 minutes with diffusion pump).

7) The ampoules are removed from the vacuum line and allowed to warm at room temperature and then placed in preheated block at 485°C for 40 minutes.

3.6 Analysis

The samples are analyzed for D/H ratio on a mass spectrometer against V.SMOW (Vienna Standard Mean Ocean Water). Slush of –80°C is used to remove the impurities. Appropriate internal laboratory standards are also used to report values against international standard VSMOW [19].

3.7 Calculation and Results

The data obtained from the analysis of D/H is extrapolated by using the Microsoft Excel computer programme for the calculation of the milk intake [19]. The underlying assumption is that isotope is distributed rapidly within a single compartment that are the mother's and the baby's total body water. A change in the ^{2}H enrichment in the mother is the function of milk and non milk water fluxes, while maternal total body water volume is regarded as constant. The appearance and subsequent disappearance of ^{2}H in the infant's body water is a function of milk water intake, other water intake and water losses. Provided that milk intake increases or decreases proportionately, the infants total body water may change during the measurement period. A model is described in Fig. 3. Compartmental analysis of this system leads to the expression [20].

$$\frac{Eb(t)}{Em(0)} = \frac{fbm}{Vb} \quad \frac{1}{kbb - Kmm} \quad (e^{-kmmt} - e^{-kbbt}) \text{----------(1)}$$

Where Em and Eb are ^2H enrichment values in the mother's saliva and baby's urine, corrected for enrichment in the predose samples. V_b is the baby's total body-water volume, while kmm and kbb are fractional rate constants. Fbm is the total milk intake of the infant. The values of D/H ratio of the samples obtained from the analysis of samples on mass spectrometer into the computer microsoft excel programme were fed. Which calculates the milk intake as follows

- The mean value of ^2H enrichment obtained from the analysis of samples collected from mother and her exclusively breast fed infant over a 14 days period are subtracted from the mean pre dose values.
- Curves are drawn by the computer showing a decrease in the enrichment of mother samples and increase enrichment in the baby samples (Fig. 2).
- From the curve Kmm, Em(o) and Kbb are obtained. Kmm and Em(0) are the constants (slopes and intercepts) of the mother's mono exponential ^2H disappearance curve.
- Values for fbm/Vb can be found by measuring Em(t) and Eb(t) (values above the background) and applying them to compartmental analysis [21].
- Vb, total body water of the baby, can be calculated from Friis- Hansen [22] formula for children less than 1 year of age. which is as follows

  $\mathbf{Vb = 0.92W^{0.8}}$ where W is the weight (kg) and Vb is the volume (litre).----- **(2)**
- Fbm is obtained by multiplying fbm/Vb by an estimate of Vb. Since Fbm represents only the free water in milk and does not include water from the oxidation of milk solids. Milk-intake/day (M) is obtained from the expression by assuming that milk is 87.1% water

M = fbm/0.87----------**(3)**

Other parameters can also be calculated from the data available from the above methodology. e.g, estimates of breast milk output, maternal body composition (total lean body mass and total fat) and estimates of non-milk water intake (Fbo). A work sheet of excel computer programme is shown in Fig. 6.

Fig. 6 A typical work sheet of computer in excel programme

MOTHER'S DATA

age	22.00	years
Weight	50.00	kg

BABY'S DATA

age	1.00	months
start weight	3.85	kg
final weight	3.94	kg

DOSE DATA

Dose	10.00	g
Dose diluted	0.10	g
Tap water	250.00	g
ppm in dil dose	300	

(1) Data for mothers saliva or milk

time	ppm	ppm
0.00	147.63	147.90
1.00	273.39	276.34
2.00	249.54	259.12
3.00	232.30	230.64
4.00	218.89	219.30
13.00	159.95	157.25
14.00	155.56	159.79

mean-predose	sigma	cv	ppm calc	chi squared
			156.77	
127.10	2.09	1.64	128.16	0.00
106.57	6.77	6.35	104.77	0.00
83.70	1.18	1.41	85.66	0.00
71.33	0.29	0.41	70.03	0.00
10.84	1.91	17.64	11.42	0.00
9.91	2.99	30.16	9.34	0.00
			sum =	**0.01**

(2) Data for baby's saliva or urine

time	ppm	ppm
0.00	150.38	150.43
1.00	177.67	183.17
2.00	200.48	204.24
3.00	212.47	210.00
4.00	216.94	211.89
13.00	175.19	174.20
14.00	171.44	165.25

mean-predose	sigma	cv	Body Water	del calc	chi squared
			2.70	0.00	
30.01	3.89	12.95	2.71	32.23	0.01
51.95	2.66	5.12	2.71	50.37	0.00
60.82	1.75	2.87	2.72	59.07	0.00
64.01	3.57	5.58	2.72	61.62	0.00
24.28	0.70	2.88	2.75	22.66	0.00
17.94	4.38	24.40	2.76	19.22	0.01
				sum =	**0.02**

MOTHER'S COMPOSITION

D space	23.92	kg
Lean Mass	31.51	kg
Body fat	18.49	kg
fat	36.98	%
water intake	4.82	kg.day-1

KINETIC DATA

Cm(0) =	156.77	ppm	**Milk vol**	**0.82**	**kg.day-1**
k(mm) =	0.20	day-1	F(bo)=	0.03	**kg.day-1**
k(bb) =	0.29	day-1	**Total error**	**0.16**	
F(bm) =	0.71	kg.day-1			
k(bm) =	0.03	day-1			

4. ADVANTAGES AND DISADVANTAGES OF DIFFERENT METHODS.

Test weighing method

⇒ Test weighing in carefully controlled setting can give accurate measurement of breast milk intake but accuracy may be difficult to achieve in non clinical setting,

⇒ There is a great need for the use of sophisticated , high precision balances in the inappropriate circumstances existing in the technologically under developed societies, where feed volume is small and frequent.

⇒ Test weighing method gives high variation in the breast milk intake from day to day, within the same individual, and between the individuals of different societies.

⇒ One-day test weighing with good balance shows less coefficient of variation then the test weighing of four days.

⇒ The test weighing method require high levels of supervision and is labour intensive in the field.

⇒ As the volume of breast milk consumed is the result of an interaction between mother and infant any disruption will alter this relationship and have an unknown effect on habitual intake.

⇒ Test weighing also interrupts usual feeding routine

⇒ Test weighing is mostly used for small animals.

All these factors reduce the precision of test weighing procedure.

Flow meter method

⇒ This method provides an exact estimation of milk out put/ flow rate as the child feeds and simultaneous composition study of the milk.

⇒ There is great potential for such a device in a metabolic ward, but for community studies the technological complications reduce its use.

Deuterium Dilution Method

D_2O dilution technique is more precise than the test weighing and flow meter method for the following reasons.

⇒ This method through the sophisticated instrument, has the advantage of being a field method and can be performed without disrupting the normal routine.

⇒ Deuterium is a non radioactive and readily available in the form of D_2O.

⇒ The D_2O method is a non invasive.

⇒ D_2O method imposes less constraints on the mother and child than test weighing and does not disrupts the normal routine of the mother or the baby.

⇒ D_2O method gives an estimate of milk that is absorbed rather than that is ingested only.

⇒ The tracer technique cause minimal interference with feeding behavior .

⇒ Deuterium samples can be stored for a long time as compared to the tritium labeled samples before analysis.

⇒ Estimate can be made over a greater number of days, and errors, which are associated with test weighing can be avoided in the tracer technique.

⇒ Basic measurement is a value related to the effects of all feed consumed in a period of 14 days, whereas test weighing involves the summation of quantities found in the measurement of individual feed.

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