J. Clin. Chem. Clin. Biochem.
Vol. 26, 1988, pp. 715–721
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Berlin · New York

# Measurement of $D_2O$ Concentrations at Tracer Levels in Small Samples Obtained from Paediatric Patients

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(Received June 10/August 26, 1988)

*Dedicated to Prof. Dr. J. R. Bierich on occasion of his 68th birthday*

**Summary:** A micro-method is described for the determination of trace amounts of $D_2O$ in serum, saliva and urine, requiring 100 µl of sample. $H_2O/D_2O$ are rapidly isolated from serum, saliva or urine by vacuum distillation. $D_2O$ concentrations in $H_2O$ are detected by infrared spectroscopy utilizing the integrated absorption of the OD-bond at 2510 $cm^{-1}$ in the range of 2675 to 2460 $cm^{-1}$. Calibration is done using standard solutions of $H_2O/D_2O$.

The separation of $D_2O/H_2O$ is necessary because of the varying background absorption of the sample. The recovery of $D_2O$ after distillation is 100%.

The absolute error is 25 mg/kg ($\pm$ 2 s) of $D_2O$ leading to a precision of 17.5% at 150, 2.8% at 1000, 1.3% at 2000, 0.9% at 3000, 0.7% at 4000 and 0.5% at 5000 mg/kg of $D_2O$.

The usefulness of the method is evaluated by 9 determinations of total body water and water turnover in 7 healthy subjects drinking a mixture of $H_2O/D_2O$.

Total body water related to body surface is 23.3 $\pm$ 0.9 $l/m^2$ in males and 20.5 $\pm$ 1.5 $l/m^2$ in females.

Water turnover is determined by analysing the decrease of $D_2O$ concentrations in blood between 6 and 31 days. A mean of 1.53 $\pm$ 0.13 $l/m^2 \cdot d$ in males in 1.48 $\pm$ 0.17 $l/m^2 \cdot d$ in females was found.

## Introduction

Several methods have been established for measuring the water compartment and water turnover of the body. Early estimates were made (1, 2) by measuring the difference between the wet and dry weights of human bodies. Indirect methods used the specific gravity of the body or the alteration of serum osmolarity after infusion of hypertonic saline solution (3).

In the 20th century, methods were introduced using tracer substances. Compounds such as urea (4), thiourea (5), sulphonamides (6), and antipyrine (7) have been used but these methods are inaccurate because the tracer substances are eliminated at individual rates thus producing an undefinable individual error in the estimation of body water.

With the discovery of deuterium, the non-radioactive isotope of hydrogen, methods using the dilution of deuterium oxide in the body water compartment have been well established.

*Lucke & Harvey* found that $D_2O$ permeates biological membranes as fast as $H_2O$ (8). *Edelman* showed that 2 hours after application, the concentration of $D_2O$ in $H_2O$ reaches a plateau of identical values in all body tissues (9).

The toxicity of $D_2O$ has been widely investigated. During short term application, $D_2O$ volume fractions of about 0.40 are tolerated without any pathological effect (10–13). During long term studies, however, fertility appears to be impaired if $D_2O$ concentrations exceed a volume fraction of 0.20 (14–16). This effect

is fully reversible. *Murphy* et al. (17) found a significant depression of fibroblast growth rate at $D_2O$ concentrations above a volume fraction of 0.20. Thus, during short term evaluation, tracer concentrations of $D_2O$ should not exceed the volume fraction of 0.01 or 10 g/kg.

Many methods have been described for determining trace levels of $D_2O$ in body fluids. All, however, are time-consuming and require large amounts of serum or other body fluids. In order to apply the $D_2O$ dilution method to paediatric problems we have developed a fast and accurate micromethod requiring not more than 100 µl of serum.

## Materials and Methods

### Distillation

$H_2O$ and $D_2O$ are separated from other sample components by vacuum distillation at 1 Torr and subsequent sublimation at −190 °C using the apparatus shown in figures 1 and 2. The

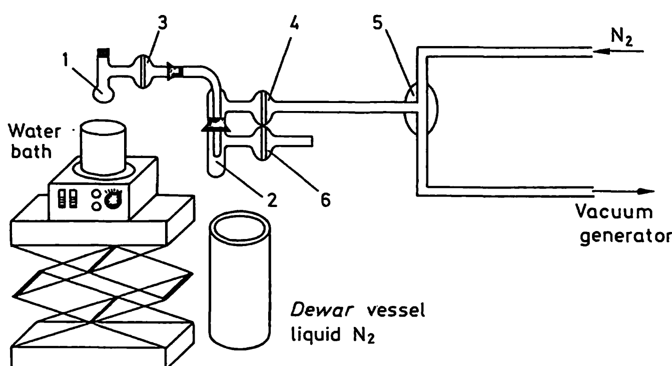


Fig. 1. Glassware used for distillation.
1: bulb, 2: tube, 3, 4, 6: two way stop cocks, 5: three way stop cock, 7: heated water bath, 8: *Dewar* vessel containing liquid $N_2$. An aliquot of the serum which is to be distilled is placed in bulb 1 hanging above the water bath. Using stop cock 5, either the vacuum pump or the supply of dried pure $N_2$ may be connected to the glassware.

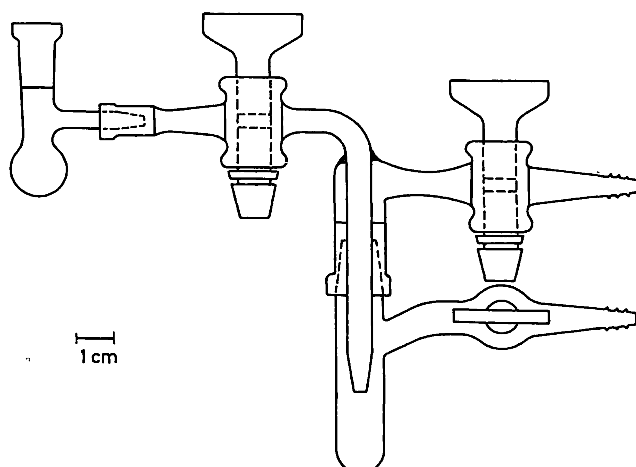


Fig. 2. Details of figure 1.

apparatus, in the position shown in figure 1, is evacuated by connecting to a vacuum pump and is subsequently rinsed with dry $N_2$. The glassware is lowered so that tube 2 is partially immersed in liquid nitrogen. After placing a sample aliquot (minimum quantity about 100 µl) in bulb 1, tube 2 is evacuated by closing stop cock 3. When valve 4 is closed and valve 3 opened, the gas pressure in the glassware is lowered to about 20% as compared to atmospheric pressure because the volume ratio of bulb 1 to tube 2 is 1:4. On repeating this procedure three times, $H_2O$ and $D_2O$ are evaporated at 1 Torr and sublimed into tube 2.

During evaporation, the temperature of both sample and bulb 1 falls below 0 °C. Therefore, the water bath is positioned touching bulb 1 in order to liquify and subsequently distil the frozen sample to dryness.

After raising the apparatus out of the liquid nitrogen, the sublimate in tube 2 is melted to a clear liquid which is aspirated into a 1 ml syringe and transferred to the $CaF_2$ cell of the spectrometer.

### Detection of $D_2O$ concentrations

The concentration of $D_2O$ in water is measured using a double beam infrared spectrometer (Perkin Elmer, Bodenseewerke, Überlingen, FRG, type 598) connected to a 64 kbyte computer employing two floppy disk cartridges (Perkin Elmer Infrared Data Station 3600, software "Quant«'). A hard copy of the display is generated by a Perkin Elmer printer type 660.

Cells equipped with $CaF_2$ windows (Perkin Elmer Bodenseewerke) are used with a volume of 90 µl and path length of ca 0.1 mm. A temperature of 20.0 ± 0.01 °C is maintained beginning 2 hours before starting the analyses.

Concentrations of $D_2O$ are measured by transferring the distillate into the cell mounted in the sample beam, and tap water into the cell mounted in the reference beam. The integrated absorption of the OD bond at 2510 $cm^{-1}$ is recorded in the range 2460–2675 $cm^{-1}$. Standard curves are constructed by measuring the absorption of 3 standard solutions containing 150 (tap water), 2640 and 5120 mg/kg $D_2O$ prepared by dilution of $D_2O$, volume fraction 0.998 (Aldrich Janssen, Nettetal, FRG: goldlabel, 15188-2). After filling the cells with sample or standard solution, the temperature is allowed to equilibrate for 5 minutes and the mean absorption of 5 subsequent scans is calculated.

### In vivo measurements of total body water and water turnover in man

To seven healthy volunteers, 4 males and 3 females, 200 ml of a mixture of $D_2O/H_2O$ with known content of $D_2O$ was given orally. Table 2 shows anthropometric data of the subjects and the dose of $D_2O$. No food or fluid intake was allowed one hour before and three hours after drinking the mixture. The subjects remained in a supine position. Two subjects, P. L. and R. S. were assayed twice within 5 months.

Blood samples were collected from an antecubital vein every 5 minutes during the first hour after oral load and every 15 and 30 minutes during the second and third hour. Distillation and analysis of the sample was performed using 100 µl of serum.

## Calculations

Total body water was then calculated using the equation:

$$TBW = \frac{V}{c_{eq} - c_0} \qquad \text{(Eq. 1)}$$

TBW : total body water [l],
V : consumed volume of $D_2O$ at 37 °C [l],
$c_o$ : $D_2O$ concentration before administration (= 150 mg/kg)
$c_{eq}$ : $D_2O$ concentration after homogeneous distribution in body water (usually 3 h after administration) [mg/kg]

The lean body mass was calculated using:

$$LBM = \frac{TBW}{C} \quad \text{(Eq. 2)}$$

LBM : lean body mass [kg],
TBW : total body water [l],
C : water content of the lean body mass, usually 0.726 l/kg (18).

The compartment of body fat was calculated using:

$$F = W - LBM \quad \text{(Eq. 3)}$$

F : body fat [kg],
W : body weight [kg],
LBM : lean body mass [kg].

For estimation of the water turnover, a second and a third blood sample was taken from 6 of the 7 probands one to four weeks later. The elimination rate of $D_2O$ $k_{20}$ was then calculated, using

$$k_{20} = \frac{\ln(c_{t_1} - c_o) - \ln(c_{t_2} - c_o)}{t_2 - t_1} \quad \text{(Eq. 4)}$$

$k_{20}$ : elimination rate of $D_2O$ [$h^{-1}$],
$c_o$ : $D_2O$ concentration of water (= 150 mg/kg)
$c_{t_1}$, $c_{t_2}$ : $D_2O$ concentrations at $t_1$ and $t_2$ [mg/kg]
$t_1$, $t_2$ : time of blood collection after homogeneous distribution of $D_2O$ in body water [h].

The biological half time $\tau_{1/2}$ is:

$$\tau_{1/2} = \frac{\ln 2}{k_{20}} \quad \text{(Eq. 5)}$$

The rate of daily water turnover is:

$$R_{H_2O} = k_{20} \cdot TBW \cdot 24\ h \quad \text{(Eq. 6)}$$

$R_{H_2O}$ : daily water turnover [l],
$k_{20}$ : coefficient of elimination according to Eq. 4 [$h^{-1}$],
TBW : total body water [l].

## Results

### Recovery of $D_2O$ after distillation

The concentration of $D_2O$ of four solutions containing 1600, 2800, 4300 and 5300 mg/kg $D_2O$ were measured before and after distillation. The recovery of $D_2O$ over the whole range is 99.90 ± 0.03%.

### Calculated precision of the method

Six calibration solutions containing 150 (= demineralized $H_2O$), 930, 1930, 2940, 3950, and 4950 mg/kg $D_2O$ in $H_2O$ were measured ten times to determine the tolerance intervals for $\alpha = 0.05$ and $\alpha = 0.01$. The exact values of the ranges of tolerance were calculated by regression analysis. The tolerance interval showed a constant level of 25 mg/kg in the whole range revealing a relative error of 17.5% at 150, 2.8% at 1000, 1.3% at 2000, 0.9% at 3000, 0.7% at 4000 and 0.5% at 5000 mg/kg.

### Measured precision of the method

Aliquots of two sera containing 1160 and 6160 mg/kg of $D_2O$ were stored at −20 °C and analysed 13 times in 13 days. The concentrations of $D_2O$ found were 1160 ± 24 mg/kg and 6161 ± 26 mg/kg giving an interassay variation of 2.1% for the lower and 0.4% for the higher concentration of $D_2O$.

These results are within the range of the predicted values.

### Necessity of serum and urine distillation before infrared analysis of $D_2O$

*Serum samples*

In undistilled native sera inconsistent absorbances are found not only in different persons but also in samples drawn from the same subject at different times, leading to varying and falsely high apparent concentrations of $D_2O$, ranging from 50 to 78 mg/kg. These samples give identical values (150 ± 10 mg/kg) after distillation prior to spectroscopy. This indicates that the different apparent values of $D_2O$ concentrations found in undistilled sera are due to varying background absorptions rather than to different concentrations of $D_2O$.

*Urine and saliva samples*

The detection of $D_2O$ concentrations in urine or saliva is accurate when the sample is distilled before analysis. Table 1 shows the results of seven different urine samples measured before and after distillation. The

Tab. 1. Concentrations of $D_2O$ measured in 7 different urine samples before and after distillation.

| No. | Density | Apparent $D_2O$ concentration found before distillation [mg/kg] | $D_2O$ concentration found after distillation [mg/kg) |
|---|---|---|---|
| 1 | 1030 | 1440 | 150 |
| 2 | 1030 | 1270 | 150 |
| 3 | 1030 | 1460 | 150 |
| 4 | 1029 | 1630 | 170 |
| 5 | 1030 | 2060 | 160 |
| 6 | 1028 | 1260 | 140 |
| 7 | 1024 | 610 | 170 |
| $\bar{x}$ | | 1390 | 160 |
| s | | 44 | 10 |
| CV [%] | | 31.6 | 6.3 |

absorbance of the undistilled urine is not constant, giving falsely high and scattered values. The distilled urine, however, reveals precisely the normal concentration of 150 mg/kg $D_2O$. The data for saliva are not shown, but the absorbance of distilled saliva is identical to the absorbance of demineralized water.

## The measurement of total body water and water turnover

Figure 3 shows a typical curve of the appearance of $D_2O$ in blood within the first three hours after oral intake. The concentrations are related to the concentration of $D_2O$ at equilibrium $c_{eq}$. A plateau is reached 90 minutes after intake, indicating the stable and homogenous distribution of $D_2O$ in the water compartment.

### *Total body water*

Table 2 shows the concentration at equilibrium after homogeneous distribution of $D_2O$ in the body water for all volunteers.

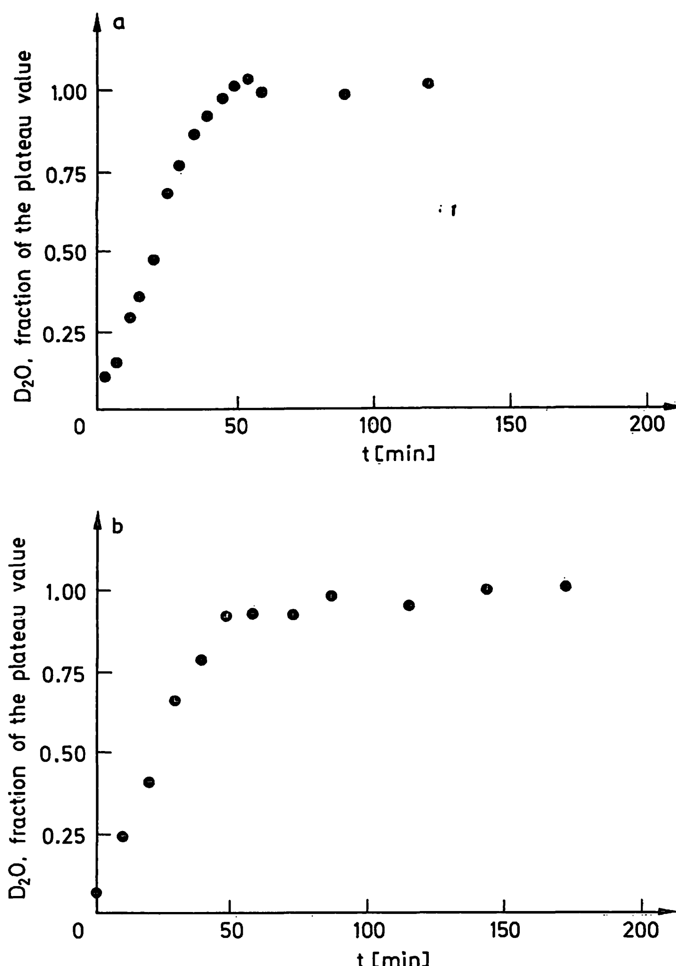


Fig. 3. Kinetics of $D_2O$ appearance in serum after oral application of $D_2O$ for 2 out of 7 subjects (a, b). The concentrations are given as fractions of the plateau concentration reached after 2—3 hours (y-axis). The x-axis shows time after oral intake in minutes.

In Table 3 the calculated values for total body water, body fat and lean body mass using equations 1—3 and values related to body weight and surface are listed. The amount of body water related to body surface appeared to be fairly constant: 23.3 ± 0.9 $l/m^2$ in males and 20.5 ± 1.5 $l/m^2$ in females. The

Tab. 2. Age, weight, height, body surface (according to *Du Bois* (30)) and administered amount of $D_2O$ of 7 healthy volunteers. The $D_2O$ concentration in serum ($c_{eq}$) after complete, stable and homogeneous distribution in the body water compartment and $t_{1/2}$, the time to reach 0.5 $c_{eq}$, are shown.

| Name | Sex | Age [a] | height [cm] | Weight [kg] | Body surface [m²] | $D_2O$ [ml] | $c_{eq}$ [mg/kg] | $t_{1/2}$ [min] |
|---|---|---|---|---|---|---|---|---|
| C. S. | ♀ | 23 | 167.0 | 56.0 | 1.63 | 48.0 | 1500 | 12.7 |
| S. E. | ♀ | 23 | 168.5 | 58.5 | 1.67 | 44.2 | 1420 | 16.2 |
| I. S. | ♀ | 30 | 168.5 | 66.7 | 1.76 | 57.2 | 1880 | 10.7 |
| M. K. | ♂ | 23 | 181.4 | 67.6 | 1.87 | 43.2 | 1090 | 10.3 |
| D. R. | ♂ | 27 | 180.5 | 66.5 | 1.85 | 69.7 | 1740 | 10.3 |
| P. L. 1 | ♂ | 30 | 178.4 | 77.0 | 1.95 | 46.6 | 1220 | 20.8 |
| P. L. 2 | | 31 | 177.3 | 75.2 | 1.92 | 89.4 | 2210 | 23.9 |
| R. S. 1 | ♂ | 24 | 180.0 | 65.0 | 1.83 | 59.3 | 1570 | 9.6 |
| R. S. 2 | | 25 | 178.5 | 67.0 | 1.84 | 68.5 | 1800 | 12.4 |

Tab. 3. Total body water (TBW), lean body mass (LBM) and fat mass (FM) in absolute values as well as related to weight and body surface of the probands.

| Name | Sex | TBW [l] | TBW [%] | TBW [l/m²] | LBM [kg] | LBM [%] | LMB [kg/m²] | FM [kg] | FM [%] | FM [kg/m²] |
|---|---|---|---|---|---|---|---|---|---|---|
| C.S. | ♀ | 35.6 | 63.5 | 21.8 | 49.0 | 87.5 | 30.1 | 7.0 | 12.5 | 4.3 |
| S.E. | ♀ | 34.8 | 59.5 | 20.8 | 47.9 | 81.9 | 28.7 | 10.6 | 18.1 | 6.4 |
| I.S. | ♀ | 33.1 | 49.6 | 18.8 | 45.6 | 68.4 | 25.9 | 21.1 | 31.6 | 12.0 |
| x̄ | | | 57.5 | 20.5 | | 79.3 | 28.2 | | 20.7 | 6.4 |
| s | | | 7.2 | 1.5 | | 9.8 | 2.1 | | 9.8 | 4.0 |
| CV | | | 12.4% | 7.4% | | 12.4% | 7.6% | | 47.4% | 61.8% |
| M.K. | ♂ | 45.9 | 67.8 | 24.5 | 63.2 | 93.5 | 33.8 | 4.4 | 6.5 | 2.4 |
| D.R. | ♂ | 43.8 | 65.9 | 23.6 | 60.3 | 90.7 | 32.6 | 6.2 | 9.3 | 3.4 |
| P.L. 1 | ♂ | 43.5 | 56.6 | 22.3 | 59.9 | 77.8 | 30.7 | 17.1 | 22.2 | 10.4 |
| P.L. 2 | | 43.4 | 57.7 | 22.6 | 59.8 | 79.5 | 31.1 | 15.4 | 20.5 | 8.0 |
| R.S. 1 | ♂ | 41.8 | 64.3 | 22.8 | 57.6 | 88.6 | 31.5 | 7.4 | 11.4 | 4.0 |
| R.S. 2 | | 41.5 | 61.9 | 22.6 | 57.2 | 85.4 | 31.1 | 6.2 | 9.3 | 3.4 |
| x̄ | | | 63.5 | 23.3 | | 87.5 | 32.2 | | 12.5 | 4.9 |
| s | | | 4.6 | 0.9 | | 6.5 | 1.3 | | 6.4 | 3.0 |
| CV | | | 7.3% | 4.0% | | 7.4% | 4.1% | | 51.4% | 61.1% |

Tab. 4. Decrease of $D_2O$ concentrations after equilibrium during 5.9 to 32.0 days. Calculated values of the coefficient of elimination $k_{20}$ and water turnover $R_{H_2O}$ using equation (5) and (6) are shown.

| Name | Sex | Time after equilibrium [h] | $D_2O$ concentration [mg/kg] | $k_{20}$ [h⁻¹] | $R_{H_2O}$ [l/d] | $R_{H_2O}$ [l/d · m²] |
|---|---|---|---|---|---|---|
| I.S. | ♀ | 0 | 1500 | | | |
| | | 328 | 633 | 0.00295 | 2.5 | 1.5 |
| | | 594 | 362 | 0.00332 | 2.8 | 1.7 |
| S.E. | ♀ | 0 | 1420 | | | |
| | | 140.5 | 1033 | 0.00259 | 2.2 | 1.3 |
| | | 279.5 | 757 | 0.00270 | 2.3 | 1.4 |
| x̄ | | | | | | 1.48 |
| s | | | | | | 0.17 |
| CV | | | | | | 11.6% |
| M.K. | ♂ | 0 | 1090 | | | |
| | | 140.5 | 795 | 0.0268 | 3.0 | 1.6 |
| | | 311.5 | 551 | 0.00278 | 3.1 | 1.7 |
| P.L. 1 | ♂ | 0 | 1220 | | | |
| | | 233.5 | 763 | 0.00239 | 2.5 | 1.3 |
| | | 358.5 | 575 | 0.00293 | 3.1 | 1.6 |
| P.L. 2 | | 0 | 2210 | | | |
| | | 351 | 933 | 0.00276 | 2.9 | 1.5 |
| | | 519 | 644 | 0.00277 | 2.9 | 1.5 |
| R.S. 1 | ♂ | 0 | 1570 | | | |
| | | 249 | 881 | 0.00267 | 2.7 | 1.5 |
| x̄ | | | | | | 1.53 |
| s | | | | | | 0.13 |
| CV | | | | | | 8.2% |

coefficients of variations are 4.0% in males and 7.4% in females.

In two subjects (P.L. and R.S.) body water was measured twice during 5 months. In both subjects identical values were found (P.L. 43.5 and 43.4, R.S. 41.8 and 41.5 liters). The body weight however, had changed in the mean time (P.L. W = −1.8 kg, R.S. W = +2.0 kg). Changes in the calculated values of the fat compartment (P.L. body fat = −1.7, R.S. body fat = +2.4 kg) correspond to these values. Thus, apart from the error of precision, the changes in body weight seem to be due solely to the changes of the body fat.

*Water turnover*

Table 4 shows the decrease of $D_2O$ concentrations in six of seven volunteers measured within 5.9 to 30.6 days after oral intake of $D_2O$. The coefficients of elimination $k_{20}$ and the water turnover both in absolute values and related to the body surface are listed. In males, a mean of 1.53 ± 0.13 (CV = 8.2%), in females, a mean of 1.48 ± 0.17 $l/m^2$ (CV = 11.6%) were found.

## Discussion

### Distillation of serum and urine

As shown above distillation of serum, saliva and urine samples is necessary to eliminate all background effects.

Most procedures described in the literature for the separation of $H_2O/D_2O$ from the serum use vacuum distillation. These methods take between one and five hours (13, 19–22) requiring a minimum amount of serum between 1.0 and 10 ml (13, 19–23). Furthermore, most of these methods need special technical equipment. The highest precision reported in the literature is about 1% (13, 20, 21, 23).

In contrast, we require not more than 100 µl of sample. The distillation may be performed fairly quickly taking about 10 minutes from the first rinsing with nitrogen until aspiration of the distillate. The recovery of $D_2O$ after distillation (99.90 ± 0.03%) is at least as high as that of previously reported methods. This precise recovery indicates that the distillation is not influenced by the different masses of normal and heavy water.

The sensitivity and precision of the method were not separately analysed, due to an inbuilt error in the spectroscopy.

### Detection of $D_2O$ concentrations

Several methods for the determination of the content of $D_2O$ in serum and urine samples have been described in the literature. However, methods using the absorption of the OD bond at 1668 nm or the absorption of slow moving neutrons cannot be used when $D_2O$ concentrations are below 5000 mg/kg, because of their low sensitivity (24). They may only be used at concentrations exceeding a $D_2O$ volume fraction of 0.20.

On the other hand, the precision and sensitivity of methods such as NMR, gas chromatography, and/or mass spectrometry are similar to our method ((24, 25), personal communication Department of Chemistry, University of Tübingen). *Schloerb* et al. (26) described a method using the sinking rate of a drop falling in *o*-fluorotoluene which was simple and accurate (CV 0.6% at 1500 mg/kg $D_2O$).

The measurement of the infrared absorbance of the OD bond at 2510 $cm^{-1}$ has been used at least since 1956 (27). Other authors have published methods also using the absorbance of the OD bond at 2510 $cm^{-1}$ (17, 20, 22, 28, 29). The coefficients of variation range from 1 to 6% at concentrations between 1 and 10 g/kg $D_2O$.

Our method is rapid, taking not more than 10 minutes per sample. The sensitivity and precision is higher than in all previously described methods including NMR or mass spectrometry.

To achieve this high precision it is essential to keep the temperature within the $CaF_2$ cells constant in the range of ± 0.1 °C.

### In vivo measurements of total body water and water turnover

We measured the $D_2O$ concentrations in blood after oral intake of $D_2O$ to determine whether the method could be applied to physiological and clinical problems. Our results concerning the kinetics of $D_2O$ in serum after oral load, total body water and water turnover are consistent with the results reported in literature (3, 13, 26). These results and our own experience with this new method have shown that it may be safely applied to problems in neonatology where the size of samples is extremely limited.

## Conclusions

The measurement of total body water in man using deuterium oxide dilution is a well established and accurate method. Usually the concentrations of $D_2O$ are measured by infrared spectroscopy. The methods of analysis, however, are time-consuming and require large amounts of sample, thus impairing the application to paediatric problems.

Therefore, we developed a method using deuterium oxide dilution, obligatory separation of $H_2O/D_2O$ from serum, saliva or urine by vacuum distillation and measurement of $H_2O/D_2O$ by infrared absorbance. Compared with previously described methods, our method is rapid and requires very small amounts of sample. The sensitivity and the precision are at least as high as those of the reported methods.

The method may be useful in investigating children and even neonates in order to measure the total body water, kinetics of water and the lean body mass.

**Acknowledgement**

We thank Prof. Dr. *U. Kunze* †, Department of Inorganic Chemistry II, University of Tübingen, FRG, for placing the infrared spectrometer at our disposal.

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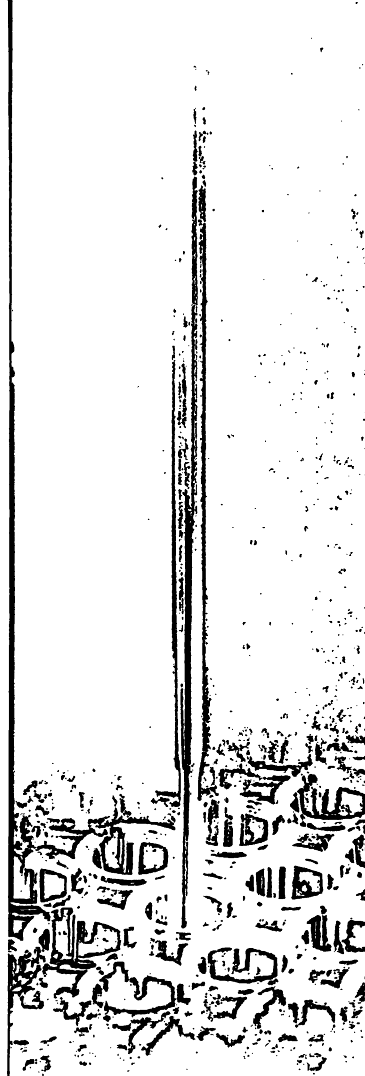



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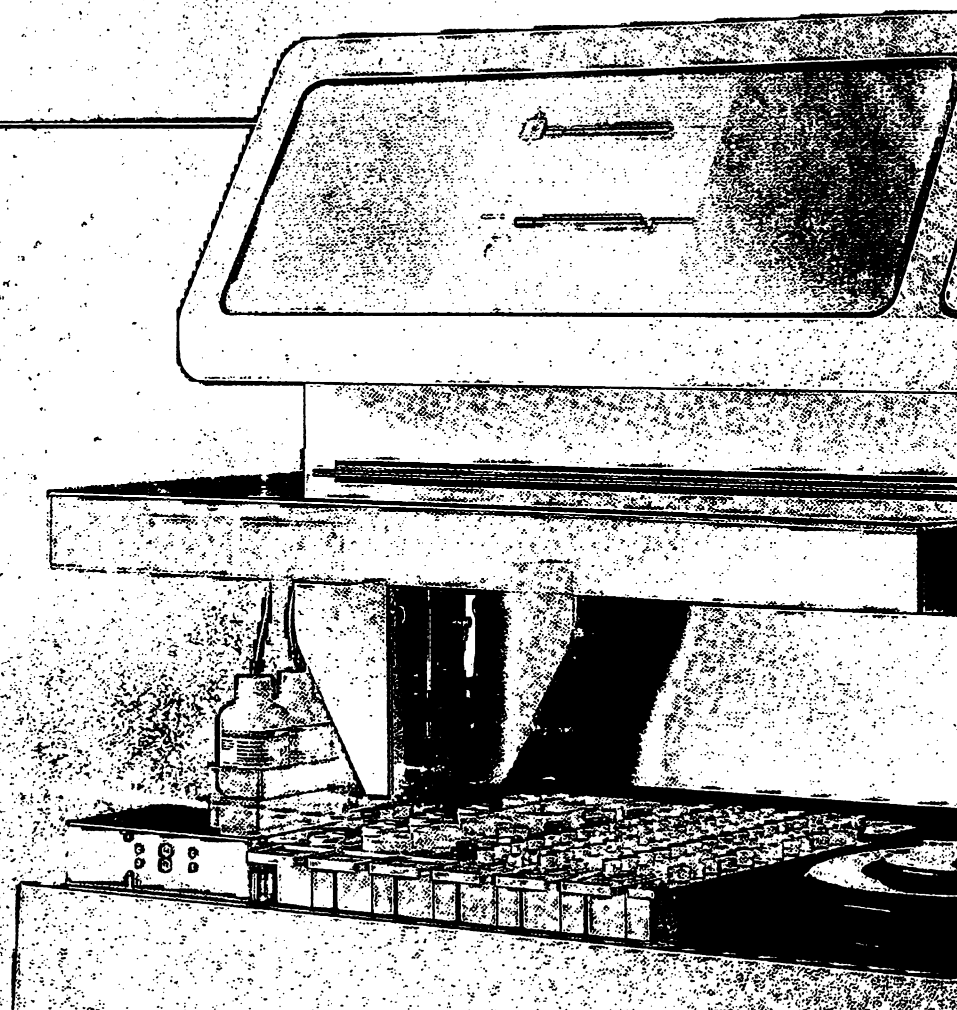

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