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Total body water and extracellular water measurements through *in vivo* dilution of D_2O and bromide as tracers

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Abstract. Large interest is into the determination of body fluids because of changes in body water in a wide variety of physiological and pathological conditions. Here methods based on dilution of tracers, are reported, for the extracellular water (ECW) and total body water (TBW) determinations on the same sample. As for ECW, bromide is used as tracer and its concentration is determined by energy dispersive X-ray spectrometry; while as for TBW, D_2O is used as tracer and HOD is determined by FT IR. Both methods represent significant improvements with respect the procedures described in the literature in terms of availability, reproducibility and accuracy.

1. Introduction

Studies of fluid balance in humans are of interest because of changes in body water in illness, malnutrition and in evaluation of the drugs distribution in pharmacokinetics studies [1]. Clinicians require a tool both for a discriminative diagnosis of fluid imbalance and for monitoring changes in water spaces in elderly patients [2].

Patients with congestive heart failure need a pharmacological treatment to restore the body fluid balance, since body weight only gives a rough estimate of body fluid content. Therefore, it would be beneficial to apply a method to identify the total body water, extracellular fluid and intracellular volume which is optimal for each patient and which diuretic therapy could aim at restoring [3].

Fluid compartments are also enlarged in obese patients, particularly with an expansion of the extracellular water (ECW) and an increased extracellular/intracellular water (ECW/ICW) ratio [4]. This ECW expansion was explained with an increased ECW/ICW ratio of fat mass [5,6] with obesity related oedema or with hormonal responses related to adipose tissue [7]. In obese subjects the weight loss by diet or by surgical treatment is more rapid in the first months and is associated with body composition and fluid distribution changes. The desirable weight loss should interest mostly fat mass with limited fat-free mass

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and body fluids variations. In fact an important fat-free mass loss and fluids unbalance are disadvantageous because often associated with muscle weakness and disability [8,9].

All classic methods to measure TBW and ECW are based on the principle of dilution; this principle requires a tracer that is not noxious, uniformly distributing in the whole unknown compartment and not interfering with water or other substances distribution of whole body.

The TBW determination has been performed by "*in vivo*" dilution method of tracer compounds: radioactive tritium oxide [10] has been extensively used, but it has been recently substituted by deuterium oxide (D_2O) [11], which is non-toxic in tracer amounts and can therefore be used in research on children and fertile women. The deuterium concentration was determined in body fluids, as HOD, by different procedures (i.e. freezing point evaluation, gas-chromatography, mass spectrometry and infrared absorption). All these methods suffer from the combination of technical and practical limitations which involve the compliance of the subjects and the quantitative determination which must be reproducible within reasonable error limits.

To evaluate ECW inulin, manitol, thiocyanate, thiosulphate, bromide but also compounds containing radioisotopes ^{82}Sr, ^{34}S, ^{24}Na and ^{82}Br have been used as tracers [12]. Bromide is the most used one in estimating ECW, because it is neither radioactive nor noxious in the small quantities usually given to patients [13]. Bromide has almost the same distribution as chloride and the advantage of good absorption, only moderate penetration into cells and slow excretion [12]. Yet, the bromide space determination represents a good measure of extracellular fluid and is used like the reference method for the validation of other fast methods not based on dilution principle, such as Bioelectrical Impedance Analysis (BIA) [14].

The critical point in the bromide space determination is the accuracy of the measure of the plasma bromide concentration, which is extremely low. Several techniques have been employed for bromide determination in plasma, including the colorimetric bromate-rosaniline method [15], Neutron Activation Analysis (NAA) [16], X-ray-emission by protons (PIXE) [17] and photons (XRF) [18] and bromide ion chromatography [19]. NAA and PIXE are very long time consuming techniques and require expensive equipment which is not routinely available in a medical laboratory, the anion-exchange chromatographic method needs giving an high oral dose of bromide to patients [20]. EDXRS seems to be the most advantageous technique to routinely measure the bromide concentration.

Here we describe a method for both TBW [21] and ECW [22] evaluations through D_2O and bromide determinations in plasma, respectively.

2. Experimental

Selected subjects received orally a known doses of D_2O and NaBr (ca. 50 g of a solution containing 200 ml of D_2O in 1000 ml of H_2O solution and 35 mg/kg body weight of NaBr). Before administration of D_2O and after 3 hrs, when bromide and D_2O are uniformly distributed, 20 ml venous blood samples were drawn into syringes and transferred into heparinized stoppered tubes. The samples were centrifuged at 3000 rpm for 15 min (with ALC4236A) and frozen at $-50°C$. 1 ml and 2 ml of plasma were used for the preparation of samples for ECW and TBW evaluations, respectively.

2.1. Preparation of samples for ECW evaluation

1 ml of plasma (basal and equilibrium) was accurately mixed with 1 ml of yttrium nitrate solution (0.84%) in a Petri capsule; this solution was frozen and dried. Yttrium was chosen as internal reference and the ratios between Br and Y net intensities were used in linear calibration. The dried powders, mainly

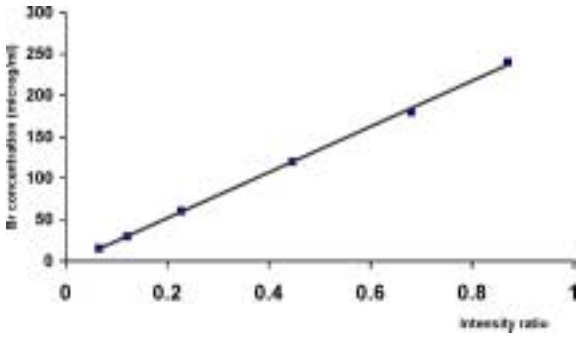

Fig. 1. Linear fit for EDXRS determinations ($r = 0.99989$).

constituted of plasmatic proteins, bromide and yttrium, were shattered for 3 minutes in an agate mortar, to obtain homogeneous powders with the same grain size; 50 mg of each powder were pressed into a 1.3 cm diameter pellet.

As the matrix and interelemental effects are relatively constant in all samples, a least squares regression analysis to relate elemental concentrations to peak ratios (Br/Y) has been used.

To obtain the calibration line 200 μl of six NaBr solutions at different concentrations (75, 150, 300, 600, 900, 1200 μg/ml) were added to basal plasma. Each standard sample, containing respectively 15, 30, 60, 120, 180 and 240 μg of NaBr, was prepared twice. In Fig. 1 the calibration plot is reported. Since the detection limit, calculated as the concentration corresponding to 3 times SD for the background counts is 5 μg/ml, the low negative value (-2.7 μg/ml) of the intercept has no influence on the accuracy of determination. Bromide concentration in both standard and unknown samples were determined by EDXRS (Energy Dispersive X-Ray Fluorescence) measurements performed with a Kevex Delta 770 spectrometer in a secondary target excitation mode in the following experimental conditions: Rh tube, Ag secondary target, tube voltage 30 kV, tube current 3.0 mA, Si(Li) detector 150 eV resolution, analysis time 300 s.

2.2. Preparation of samples for TBW evaluation

2 ml of plasma were frozen at -50°C (in a cold bath of EtOH/liquid N_2) and were then sublimed under vacuum to dryness using a diffusive pump (Edwards/Galileo) operating at 10^{-5} mbar. The sublimate was condensed in a trap immersed in liquid nitrogen giving purified water samples suitable for analysis which were stored in screw top septum vials at -20°C.

The apparatus for sublimation [21] was designed and constructed in the laboratory of the authors. The form and dimensions were optimised in order to obtain the most complete and rapid sublimation, after a number of attempts in order to obtain the most reproducible processes. In this apparatus the starting frozen material was distributed on a large surface and relatively large dimensions of transfer lines allowed a complete sublimation to dryness in ca. 5 hrs even for samples containing a large amount of solid residue (overall proteins). With smaller or different apparatus such samples required 10 hrs or more for sublimation, sometimes giving opaque sublimates and samples which were thus not suitable for IR determination. The preparation of the sublimed samples represented the key step of the determination. In fact, some relevant parameters of the sublimation, such as temperature, vacuum and determination of end point must be considered. Analyses of HOD were performed on a FT Infrared Spectrophotometer Nicolet Nexus operating under N_2 and at constant temperature of 20°C using CaF_2 cells with 0.2 mm path length.

3. Results and discussion

3.1. ECW evaluation

Energy dispersive X-ray spectrometry (EDXRS) is a powerful analytical technique, that allows for simultaneous determination, in μg/g levels, of several elements in biological samples, especially in secondary excitation mode [23]. Consequently numerous papers have been devoted to the use of EDXRS for analysing biological materials [24]. The sample and target preparation are critical steps in the analysis of such sample types. Therefore considerable effort has been made to develop and improve appropriate procedures, e.g., by making thick target pellets from dried biological materials, or by making thin-film targets obtained by low and high temperature asking, wet digestion in an open or close teflon vessels [25]. In the thin-film method the sample is chemically dissolved with acid reagents and 100–300 μl of the obtained solution are pipetted on a mylar film, dried under vacuum and analysed. The advantage of this target preparation procedure is the low matrix effect and the possibility to concentrate the solution with an appropriate ratio of sample weight to digestion solvent amount; it allows an improvement of detection limits. The calibration targets are prepared by pipetting standard solutions containing the elements to determine at different concentrations [26]. This procedure requires digestion systems, acid reagents and chemical supports which are not always available in a clinical laboratory; furthermore, the obtained targets are not easily storable for further investigation so that the calibration targets have to be prepared each time. This method was successfully applied to analyse algae, plants, hair, human tissues, blood [23,25(a),27]. The detection limits for elements with $Z \geqslant 20$ in an organic matrix, expressed as μg/ml, are in the range 0.1–0.5 [23] which correspond to 2–10 μg/g on dry weight, when 100 mg of dried sample are dissolved in 1 ml of acid. In the thick target method the specimen for the spectrometer is obtained by pressing in an IR hydraulic press 50–150 mg of dried sample powder to a 1.3 cm diameter pellet. The calibration pellets are prepared by addition of fixed amounts of the analysed elements to a reference powder. In both methods, to eliminate the geometric effects and/or small errors in the weighting or dilution steps, a fixed amount of an internal reference element (Ti, Y, Zr) is added to sample and calibration targets.

The thick targets are easier to prepare and to store than thin targets, thus the calibration specimens may be prepared at the beginning of the study and stored for further analysis, taking into account the instrumental drift. The detection limits are in the range 2–10 μg/g on dry weight basis.

In the present paper the thick target method is applied to measure the Br content in human blood to estimate the extracellular water. The published data [28] concerning this subject report the measurements of Br in blood and saliva simultaneously using stable bromine and ^{82}Br. The targets for X-ray spectrometer were obtained by placing fresh urine and blood plasma samples on a thin ash-free filter paper, dried in a vacuum. Bromine was estimated under thin film conditions with an annular ^{109}Cd source. The accuracy and precision of the method was evaluated by comparing the CBS data obtained by stable bromine to the routine method with ^{82}Br indicator. The differences in CBS estimation by ^{82}Br and EDXRF (saliva, urine and blood) are less than 10%: the advantage of stable bromine method is evident.

In the present paper the bromine was determined on blood using the thick pellets method.

Sodium bromide is administered to patients, adsorbed by body and diluted by extracellular water. The equilibrium of bromide concentration is established in blood about 3 hours after the administration [17(c)].

Extracellular water can be estimated from the Corrected Bromide Space (CBS), which was calculated according to the following formula [29]:

$$CBS = \text{Br dose (mmol)}/\text{Br in plasma (mmol/l)} \times 0.90 \times 0.95 \times 0.94,$$

where

$0.90 =$ correction factor for the distribution of bromide in the non-extracellular sites (principally red blood cells);
$0.95 =$ correction factor for the Donnan equilibrium [30];
$0.94 =$ correction factor for the concentration of water in the plasma which is about 94%.

3.2. TBW evaluation

It is well known that when H_2O and D_2O are mixed, HOD formation occurs. At low D_2O concentration the solutions consist principally of H_2O and HOD. Infrared spectroscopic analysis of dilute HOD solutions is based on the O–D stretching observed at 2500 cm^{-1} [31].

The accurate detection of the O–D stretching requires an FT IR spectrophotometer using a short path length (0.2 mm) [32]. This absorption is positioned in a free region of the spectrum but falls between the very strong CH (2800 cm^{-1}) and CO_2 absorptions (2337 cm^{-1}). The data obtained for a series of reference samples are reported in Fig. 2 and Table 1. For all samples the same range for integration between 2660 and 2400 cm^{-1} was considered. We obtained perfectly reproducible determinations on reference samples in the range of interest (110–550 ppm) even for quite dilute solutions (between 30 to 100 ppm). For the range of concentrations considered the Lambert–Beer law is strictly observed. In Fig. 2 is also reported the comparison between the data obtained for H_2O/D_2O mixtures prepared by simple dilution and the data obtained for the same solutions after sublimation, which are perfectly superimposed. These data clearly indicate that with the apparatus used for sublimation no H_2O or HOD loss occurred, as a consequence of the difference in density and vapour pressure of these two components. Figure 2 represents the

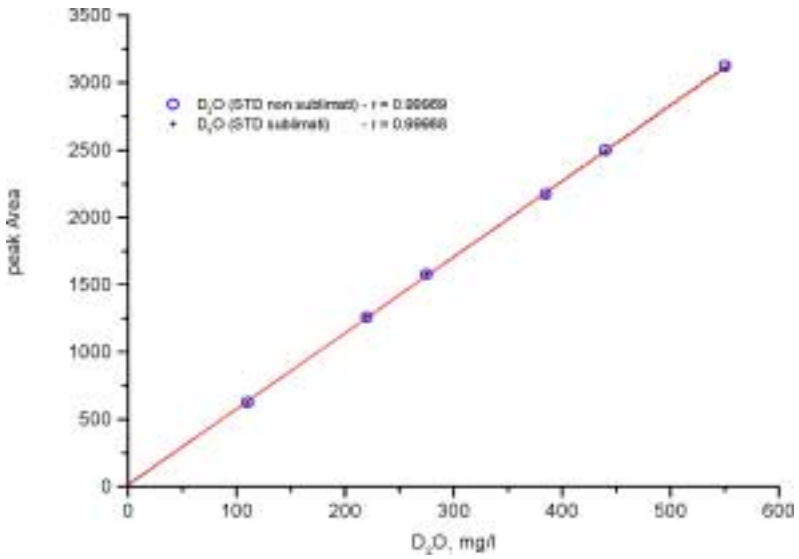

Fig. 2. Linear fit for calculations and comparison between the data obtained for H_2O/D_2O mixtures measured after dilution (○) or after sublimation (●).

Table 1

(a) Values obtained after repeated FT IR determinations on reference samples

Real concentration	Integration values – FT IR measurements on not sublimed samples		Integration values – FT IR measurements on sublimed samples	
110	629.1	average value = 627.5	619.9	average value = 624.8
	620.9		624.8	
	632.5		629.7	
220	1255.1	average value = 1257.7	1254.9	average value = 1259.5
	1264.0		1269.0	
	1253.9		1254.5	
275	1576.0	average value = 1577.8	1569.9	average value = 1576.7
	1574.9		1581.1	
	1582.5		1579.2	
385	2171.5	average value = 2171.8	2165.5	average value = 2170.2
	2174.3		2176.2	
	2169.5		2168.8	
440	2494.7	average value = 2499.4	2484.5	average value = 2492.2
	2503.1		2500.9	
	2500.5		2491.1	
550	3129.2	average value = 3123.8	3099.4	average value = 3109.6
	3127.6		3110.6	
	3114.6		3118.8	

(b) Concentration (ppm) obtained after repeated sublimations and FT IR determinations on different samples

Sample 1	236	235	232
Sample 2	272	275	269
Sample 3	264	265	260
Sample 4	339	339	330
Sample 5	364	365	365
Sample 6	262	266	264
Sample 7	390	390	390

calibration curve used for calculations. All measurements on samples were performed under the same conditions checking continuously the calibration curve. The reproducibility of the measurements falls within 1% for the HOD range concentration considered, as showed by the data reported in Table 1.

The TBW values have been finally obtained by the insertion of obtained HOD concentration in ppm into the following calculation [33]:

$$TBW = A/B \times 0.928,$$

where

$0.928 =$ correction coefficient which takes into account that D_2O is involved in H/D exchange processes into the body;
A = quantity of D_2O administered in grams;
B = concentration of HOD determined in ppm.

Table 2
ECW and TBW data obtained for a group of old CHF (congestive heart failure) women

CHF patients	ECW (ppm)	TBW (ppm)	ECW (litres)	TBW (litres)	ECW/TBW (%)
1	66	351	17.81	30.85	58
2	62	346	18.10	30.36	60
3	65	241	31.00	45.06	69
4	70	332	20.17	34.83	58
5	67	337	18.80	31.48	60
6	57	378	20.20	29.06	70
7	60	351	18.90	30.42	62
8	74	317	23.59	35.00	67
9	92	411	13.54	26.39	51
10	87	440	14.20	24.81	57
11	86	500	13.03	22.17	59
12	100	317	16.60	33.18	50
13	65	295	25.39	38.36	66
14	68	358	18.63	29.68	63

It is noteworthy that the accuracy of the value obtained for the D_2O concentration is very important, because even slight variations in this value have a large effect on the TBW value [17]. For example, a variation of 1% on a value of 300 ppm corresponds to a difference of 350 ml in TBW value.

With the methods above described, different panels of patients have been studied. Here we report as an example the data obtained for a group of old women affected with congestive heart failure (CHF). Some data are summarised in Table 2. It is noteworthy that in the healthy elderly subjects the ECW/TBW ratio ranges from 0.43 to 0.52. As Table 2 shows, elderly patients affected with CHF had an ECW expansion. As consequence ECW/TBW ratio increased presenting mean values of 60.7% [14(a)].

4. Conclusions

The current work represents a significant improvement in the ECW and TBW evaluation with respect the methods previously reported in the literature [28,32]. As for HOD determination a higher accuracy and reproducibility of the measurements can be achieved and the determinations performed on the same samples after time gave reproducible results. As for the ECW evaluation, the thick pellets are quite easy to prepare, manipulate and store, the operator has a limited contact with blood only at the drawn and centrifugation steps, so the following operations of sample preparation do not require specially trained staff.

It is noteworthy that these method are relatively inexpensive, employing instruments usually available in a clinical research laboratory and requiring minimal technical expertise in performing analysis apart from strict adherence to the procedure.

The procedure above described allows the determination of both ECW and TBW, achieved by distinct measurements, but on the same starting sample obtaining a complete evaluation of fluid compartments. Thus, the methods allow to extend the study of body fluids to further aspects of the human physiology and pathology.

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