The Use of Infrared Spectrophotometry for Measuring Body Water Spaces

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Background: The conventional method of measuring total body water by the deuterium isotope dilution method uses gas isotope ratio mass spectrometry (IRMS), which is both expensive and time-consuming. We investigated an alternative method, using Fourier transform infrared spectrophotometry (FTIR), which uses less expensive instrumentation and requires little sample preparation.

Method: Total body water measurements in human subjects were made by obtaining plasma, saliva, and urine samples before and after oral dosing with 1.5 mol of deuterium oxide. The enrichments of the body fluids were determined from the FTIR spectra in the range 1800–2800 cm⁻¹, using a novel algorithm for estimation of instrumental response, and by IRMS for comparison.

Results: The CV (n = 5) for repeat determinations of deuterium oxide in biological fluids and calibrator solutions (400–1000 μmol/mol) was found to be in the range 0.1–0.9%. The use of the novel algorithm instead of the integration routines supplied with the instrument gave at least a threefold increase in precision, and there was no significant difference between the results obtained with FTIR and those obtained with IRMS.

Conclusion: This improved infrared method for measuring deuterium enrichment in plasma and saliva requires no sample preparation, is rapid, and has potential value to the clinician.

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Total body water has been used to assess human body composition since the demonstration that a relatively fixed fraction of lean body tissue is occupied by water (1). The deuterated water (²H₂O) isotope dilution method for measuring total body water has now replaced the earlier radioactive method (³H) (2). However, the use of gas isotope ratio mass spectrometry to measure ²H₂O is expensive, requires specialist knowledge for operation and maintenance, and is somewhat tedious because the water is first transformed to hydrogen (3, 4) or equilibrated with hydrogen (5, 6).

Other methods for the determination of deuterium (²H) in water have been proposed (7–10), including infrared spectrophotometry (11–16). This latter technique has received particular attention because of its relatively high potential sensitivity, which suggests that only a small quantity of deuterium need be administered. Unfortunately, the absorption band centered at ~2500 cm⁻¹, attributable to excitation of the D–O bond, is present as a shoulder on the band at ~2130 cm⁻¹, which is attributable to the H–O bond. If a single wavelength is monitored (as is usually done with a dispersive instrument), this leads to uncertainty in the baseline reading and consequently limits the accuracy of the technique at low enrichments. In recent years, there have been considerable advances in infrared instrumentation through the application of Fourier transform infrared (FTIR) techniques. This allows rapid monitoring of the whole of the absorption region (17–19). However, these do not appear to have been used to measure enrichment in human plasma or other body fluids. Therefore, the aim of this study was to assess the precision of the new methodology and to compare results obtained from several physiological fluids with those obtained with mass spectrometry.

Subjects and Methods

Five subjects took part in this study (mean weight, 75.75 ± 4.0 kg; mean body mass index, 24.2 ± 1.70 kg/m²). Each subject arrived at the laboratory after an overnight fast (12 ± 2 h) and received an oral dose of 1.5 mol (30 g) of D₂O, 99.9% (Sigma Chemicals) followed by ~200 g of tap water [to produce an enrichment of 450–750 μmol/mol (ppm)]. The straw and dosing bottle used for the administration of the D₂O were retained and weighed to obtain the exact amount of dosing solution consumed. Samples of blood (5- to 10-mL samples collected in heparin-containing stoppered tubes) and saliva (4-mL samples collected...
were measured in the range 1800–2800 cm$^{-1}$ and described below. The infrared spectra of aqueous samples were obtained immediately before analysis, the samples were thawed, vortexed, and centrifuged. Preliminary work showed that the predose urine samples were not suitable for use as background samples for estimation of deuterium by infrared spectroscopy because of the large variations in composition of sequential urine samples. To overcome these variations, we freeze-dried weighed aliquots of the postdose urine samples and then reconstituted them gravimetrically with local tap water containing naturally occurring deuterium to provide samples of the same constituents, but without deuterium enrichment, to act as background samples for the postdose urine samples. Local tap water was also analyzed [mass spectrometry value, 147.7 μmol/mol (ppm; this value was essentially identical to values obtained from background samples of physiological fluids, e.g., saliva and plasma)].

**TREATMENT OF SAMPLES**

The saliva and plasma samples were centrifuged immediately after collection, and the supernatants were retained. All samples were stored frozen at −20 °C. Prior to analysis, the samples were thawed, vortexed, and centrifuged. The infrared spectra of D$_2$O in water obtained from the FTIR instrument is shown in Fig. 2. The signal from the deuterium-oxygen bond appears as a weak shoulder on the intense nondeuterated water band. Baseline subtraction can be performed with unenriched water as a reference, using the sample shuttle, but it is still difficult to determine the zero enrichment background with any great precision. This is demonstrated in Fig. 3, in which the zero background enrichment is subtracted from the postdose spectrum. The line at 2500 cm$^{-1}$ shows the single frequency at which most previous workers have made measurements (13, 16).

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**FTIR DATA HANDLING**

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To overcome this uncertainty in baseline positioning, a similar approach to that developed in this laboratory for gas chromatography/mass spectrometry isotope mass
ratio determination was used (20). The spectra were digitized in the region 2665–2415 cm$^{-1}$. The sample spectrum was then compared with the sum of the reference spectrum and its first two derivatives, generated numerically. The inclusion of the derivative functions allowed correction for slight spectral energy shift. Baseline subtraction was achieved by the inclusion of a linear function of light energy in the fitting procedure. This method compared the spectrophotometer outputs for sample and a reference across the whole of the relevant peak, separating the responses into that which is common to the absorption peak itself and that which is solely a property of the linear baselines beneath the peaks. Admixture of the first, second, and subsequent derivatives of the observed lineshape for the reference material allowed for nonsuperimposibility of the spectra because of instrumental drift. The equation used is:

$$S(\tilde{\nu}) = \frac{\rho \delta}{2} (\delta + 1) R(\tilde{\nu} + 1) + \rho (1 - \delta^2) R(\tilde{\nu})$$

$$+ \frac{\rho \delta}{2} (\delta - 1) R(\tilde{\nu} - 1) + A\tilde{\nu} + B$$

where $\tilde{\nu}$ is the wavenumber, $S(\tilde{\nu})$ is the sample spectrum, $R(\tilde{\nu})$ is the reference spectrum, $\rho$ is the ratio of the peak intensities, $\delta$ is the instrumental shift along the wavenumber axis between the sample and reference spectra, and $A$ and $B$ are constants related to the slopes and intercepts of the backgrounds of the spectra. In practice, the $n$ points of the digitized spectra were exported from the spectrophotometer software directly into a spreadsheet and the following matrix expression was solved:

$$\mathbf{P} = (\mathbf{R}^T \mathbf{R})^{-1} \mathbf{R}^T \tilde{\mathbf{S}}$$

where the $(n - 2)$ row vector $\tilde{\mathbf{S}}$ is made up of the digitized data for the sample, omitting the first and last points. $\mathbf{R}$ is a matrix of five columns with $(n - 2)$ rows. The first column contains the spectrum for the reference, omitting the last two readings. The second row contains the same data, but now starts at the second reading, omitting the last point; the third column contains the same data again, but omitting the first two points. The fourth column is the wavenumber, and each element of the fifth column is equal to unity. $\mathbf{P}$ is a vector of the best estimates of the parameters of the fit. Most importantly, the ratio of spectral intensities, $\rho$, can be obtained by summing the first three elements of $\mathbf{P}$. The relative amounts of deuterium in the sample can then be estimated by assuming the Beer-Lambert law to hold. In calculating the enrichment of deuterium in salivary and plasma water, we assumed (21) (and confirmed experimentally) that the hydration fraction of saliva was 99.5% ± 0.2% (in kg/L), and that of plasma, 94% ± 0.4%. The hydration fraction of the urine samples was calculated from the freeze-dried samples.

**MASS SPECTROMETRY**
Excess sample was isotopically equilibrated with hydrogen gas for 3 days, using platinum on alumina powder as a catalyst (6). The deuterium content of the resultant gas was compared with that of known calibrators, using a Sira 10 (VG Instruments) equipped with a dual inlet, and the results were converted to the SMOW/SLAP scale.

**PREPARATION OF CALIBRATORS**
Samples enriched in deuterium by known amounts were prepared by the gravimetric addition of D$_2$O (99.9%; Sigma-Aldrich) to an aliquot of natural abundance water (Cambridge tap water) and then adjustment to a known volume with tap water. A portion of this enriched sample was further diluted in Cambridge tap water to give samples enriched above natural abundance by 1000, 800, 600, 400, and 200 µmol/mol.

**STATISTICAL ANALYSIS**
Results were expressed as means and SD. Comparisons of results obtained with the two methods and between
different physiological fluids collected at the same time were made using the Bland and Altman (22) method.

ETHICS APPROVAL
The study was approved by the local ethics committee, and informed written consent was obtained from all the participants.

Results
At all levels of enrichment, the method of Bluck and Coward (20) for measuring spectral intensity was found to be more precise than the traditional integration method used by the instrument (Table 1). There was little or no significant difference between results obtained using 8, 16, 32, and 64 scans per reading (Table 2). The time taken to obtain 64 scans was 2.5 min. The precision for measurement of deuterium enrichment plasma and saliva was very similar to that of calibrator solutions (Table 3).

The results in Table 4 show that there is close agreement between both methods for mass spectrometry and infrared deuterium analysis when measurements were made in plasma, saliva, and urine (none of the comparisons were significantly different from each other; paired t-test). There also was close agreement in the enrichment between the different types of fluid analyzed (Table 5) and no significant increase in the difference between methods as deuterium enrichment increased. It was possible to analyze samples within 30 min of receiving them (including time for centrifugation).

Discussion
This study demonstrates that infrared spectroscopy, which incorporates an improved algorithm for measuring spectral intensity, can be used to measure the enrichment of D₂O in plasma and saliva rapidly and precisely (CV, 0.1–0.9% for samples with deuterium enrichment of 400 μmol/mol or more). The technique has a precision (CV, 0.1–0.9%) that is several fold better than the traditional method for measuring D₂O enrichment with FTIR (CV, 3–6% over the same range of enrichment; see text).

This study also demonstrates good agreement between the infrared and mass spectrometry measurements of deuterium enrichment in saliva, plasma, and urine. Furthermore, the differences between plasma and salivary measurements obtained with the infrared technique were similar to those obtained with mass spectrometry. However, the dose of deuterium required to achieve a CV <1% for repeated measurements of the same sample is several fold greater with the infrared method than with mass spectrometry. On the other hand, changes in background enrichment, which may occur after the administration of intravenous fluids or the drinking of distilled water, will influence the estimation of total body water by the infrared method to a lesser extent than mass spectrometry, which usually involves measurement of smaller increments in deuterium enrichment. Furthermore, the operation of mass spectrometers requires more expertise than the operation of the infrared spectrophotometer, which can yield results shortly after the samples are obtained (plasma and saliva). A particular problem with urine analysis is the presence of variable amounts of interfering urinary substances, which makes predose urine samples

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Table 1. Comparison of the CV (n = 5 separate samples) obtained using two different methods for measuring the area under the D₂O peak.*

<table>
<thead>
<tr>
<th>D₂O calibrator, μmol/mol</th>
<th>CV, %</th>
<th>Standard integrated method</th>
<th>New method*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>3.7</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>800</td>
<td>6.2</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>400</td>
<td>3.0</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>200</td>
<td>5.0</td>
<td>1.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Eight scans per reading, and five measurements per sample.

Table 2. CV (%) for the infrared method using 8, 16, 32, and 64 scans per reading.*

<table>
<thead>
<tr>
<th>D₂O calibrator, μmol/mol</th>
<th>CV, %</th>
<th>8 scans</th>
<th>16 scans</th>
<th>32 scans</th>
<th>64 scans</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.35</td>
<td>0.49</td>
<td>0.11</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.39</td>
<td>0.23</td>
<td>0.37</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>0.87</td>
<td>0.56</td>
<td>0.75</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1.4</td>
<td>1.3</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

* Five measurements per scan of calibrator solutions of D₂O. Analysis used an improved algorithm for measuring area under the curve (see text) (17).

Table 3. CV (%) for infrared measurements of enrichment of deuterium in saliva, plasma, and urine.*

<table>
<thead>
<tr>
<th>Enrichment of deuterium, μmol/mol</th>
<th>Saliva</th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.30</td>
<td>0.20</td>
<td>0.50</td>
</tr>
<tr>
<td>800</td>
<td>0.22</td>
<td>0.24</td>
<td>0.54</td>
</tr>
<tr>
<td>400</td>
<td>0.41</td>
<td>0.31</td>
<td>0.76</td>
</tr>
<tr>
<td>200</td>
<td>1.7</td>
<td>1.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Sixty-four scans per reading and five measurements per sample on 15 separate samples of each fluid. Single-cell analysis for urine and dual matched-cell analysis for saliva and plasma, using improved algorithm for measuring area under the curve (see text).

Table 4. Postdose comparison of deuterium enrichment measurements (μmol/mol) in plasma, saliva, and urine obtained with mass spectrometry and the infrared method.*

<table>
<thead>
<tr>
<th>Mean of methods (mass spectrometry and infrared)</th>
<th>Plasma, μmol/mol</th>
<th>Saliva, μmol/mol</th>
<th>Urine, μmol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>610 ± 71</td>
<td>614 ± 69</td>
<td>617 ± 67</td>
</tr>
<tr>
<td>Difference between methods (mass spectrometry – infrared)</td>
<td>6 ± 11</td>
<td>4 ± 1</td>
<td>−2 ± 12</td>
</tr>
</tbody>
</table>

* Separate samples (n = 15) were used for each fluid, with 64 scans per reading. The improved algorithm was used for measuring the area under the curve.
Differences in enrichment

5000 – 16 000 cm

of the shorter wavelength

Table 5. Postdose enrichments and differences in enrichments (bias ± SD) between physiological fluids, according to method used. 

<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Mass spectrometry</th>
<th>Infrared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>613 ± 68</td>
<td>607 ± 74</td>
</tr>
<tr>
<td>Saliva</td>
<td>616 ± 70</td>
<td>612 ± 68</td>
</tr>
<tr>
<td>Urine</td>
<td>616 ± 69</td>
<td>619 ± 65</td>
</tr>
<tr>
<td>Plasma – saliva</td>
<td>–3 ± 15</td>
<td>–5 ± 13</td>
</tr>
<tr>
<td>Plasma – urine</td>
<td>–3 ± 15</td>
<td>–11 ± 13</td>
</tr>
<tr>
<td>Saliva – urine</td>
<td>1 ± 3</td>
<td>–5 ± 10</td>
</tr>
</tbody>
</table>

* Separate samples (n = 15) of each fluid were used, with 64 scans per reading.

less suitable for background measurements than the corresponding plasma and saliva samples. Although appropriate predose urine samples can be prepared by removing the enriched water from the urine (freeze-drying) and replacing it with tap water, the process is time-consuming and unsuitable for bedside analysis. Another problem with urine analysis is that the standard calcium fluoride cells are not suitable for prolonged and repeated use, although our preliminary studies with zinc selenide cells suggest that they are a suitable alternative because they are not “attacked” by urine. Sapphire cells may also be appropriate.

Finally, there have been reports that the use of a single-cell system to measure enrichment of deuterium in urine (instead of the matched-cell system for plasma and saliva measurements) could explain the higher CV for the urinary measurements (Table 3) and the greater discrepancy between urinary and alternative fluid measurements when the infrared technique is used, compared with the mass spectrometry method (Table 5). There have been reports of temperature dependence of the infrared spectrum of both H2O and D2O in the shorter wavelength 5000–16 000 cm\(^{-1}\) region (23); therefore, some workers have used thermostatically controlled cells (13, 16). However, because the operating temperature was stable and the primary purpose of this work was to assess the technique as a simple, rapid, and inexpensive method for determination of total body water, no attempt was made to thermoregulate the sample.

In conclusion, this report describes an improved infrared analytical method for measuring D2O enrichment in physiological fluids. It is simple, precise, rapid, and of potential value to the clinician who monitors and adjusts fluid therapy on a daily basis.

References