Note

Measurement of Dietary Choline-Phospholipid Content by a Novel Phospholipase D-Triiodide Method

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Summary We developed a novel method that conveniently measures dietary choline-phospholipid content. Crude lipids extracted from dietary samples were reacted with phospholipase D from Streptomyces chromofuscus. The choline liberated from this reaction was then reacted with potassium triiodide, yielding choline periodide, which could be measured spectrophotometrically at 365 nm. This method proved to be more convenient than conventional assays, such as thin layer chromatography and high performance liquid chromatography. Our novel method is suitable for measuring many samples in single experiments.

Key Words choline, lecithin, dietary intake, phospholipase D

Choline-phospholipid, especially lecithin, is the main source of choline (1, 2), and is used therapeutically as a dietetic supplement for certain pathological conditions (3, 4). Lecithin is also delivered in drugs and in foodstuffs (5). Choline plays an important role in cell signaling, and is a precursor of the neurotransmitter acetycholine, and human studies have shown that choline deficiency is associated with liver damage (2). The human body is able to synthesize choline, but not enough to meet its requirements (1).

By analyzing the diets of approximately 400 Japanese individuals by means of the duplicate portion sampling technique, we found that limiting cholesterol intake resulted in a reduction of lecithin intake (6). We subsequently want to determine the daily intake of the choline-phospholipids (lecithin, lyssolecithin and sphingomyelin) in patients who must limit their intake of cholesterol. This can be tested using the thin layer chromatography (TLC) method. Using this method, crude lipid extracted from diets was developed on TLC plates, the area corresponding to lecithin was scraped, and the phosphorus content determined using the method described by Keenan et al. (7). However, as this procedure requires the use of strong acid and oxidant, the measurement of many samples can be detrimental to health of the researcher. This method is also not economical due to the large volume of organic solvents and many TLC plates required. Due to these limitations, we developed an alternative method.

We report in this paper a convenient method, which eliminates the need for chromatographic procedures, for measuring the choline-phospholipid content of diets.

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Materials and Methods

Samples. Crude lipids that had been extracted from Japanese dietary samples for previous studies (6) were used in this study. Lecithin (from egg yolk, purity 99%), sphingomyelin (SM, from bovine brain, purity 99%) and lyssolecithin (from egg yolk, purity 99%) were purchased from Sigma as authentic samples. These phospholipids were purified by passage through a silica Sep Pak cartridge (Waters Co, Mass.) (8). Their phosphorus content were determined by Bartlett’s method (9).

TLC method. Five to 20 μg phospholipid-phosphorus from the crude lipids extracted from the dietary samples was separated by TLC with chloroform/methanol/acetic acid/water (180: 150: 30: 10). The total phosphorus content of choline-phospholipids (lecithin, SM and lyssolecithin) was determined as well as those of individual choline-phospholipids (7). An area of adsorbent from the TLC plate was prepared as a blank control for the phosphorus calibration curve in the same way as the phospholipid-bound adsorbent.

PLase D-triiodide method. Phospholipase D (PLase D, product of Streptomyces chromofuscus; Sigma) was dissolved in 0.5 M acetate buffer pH 5.6, containing 1% KCl (820 units/mL). Five to 30 μg of phospholipid-phosphorus was added to the reaction tube, and the solvent was removed under vacuum. Next, 0.07 mL 1 M acetate buffer pH 5.6, 0.04 mL 1 M CuCl₂, 0.6 mL water and 1 mL ether were added to the tube. After sonication briefly, 0.02 mL PLase D solution and 0.27 mL water were added. After the mixture was reacted at 25°C for 30 min by shaking, ether was removed under N₂, and chloroform was added to enable the transfer of the lipids in the mixture to the organic solvent layer. The volume of the aqueous phase was measured using a pipette, and 0.5 mL was used for the choline assay.

The reaction of the liberated choline and triiodide (10) was carried out following the procedure described...
by Barak and Tuma (11). That is, 0.5 mL of the above-mentioned aqueous phase or 0.5 mL of the standard choline solutions (for calibration curve; 0 to 0.5 μmol/mL) was added to a 15 mL centrifuge tube with a narrow base. After chilling on ice, 0.2 mL of the potassium triiodide solution (15.7 g of iodine and 20 g of potassium iodide were dissolved in 100 mL of water) was mixed with the sample, which was then refrigerated overnight at 3°C. The sample was centrifuged at 2,500 × g for 20 min and the supernatant was removed, without disturbing the dark brown precipitate at the bottom of the tube. The precipitate was then dissolved in 5 mL ethylene dichloride, and the absorption was measured in a spectrophotometer at 365 nm, using ethylene dichloride as a blank. The choline content of the choline-phospholipids was estimated from a standard curve.

The results for each sample were expressed as mean ± SD. Significance of correlation between methods was tested by simple linear regression. Whether the regression line differed significantly from the line of identity was judged in two ways, i.e., whether the 95% confidence intervals (CI) for the slope included 1.0 and whether the 95% CI for the y-intercept included 0. A p value < 0.05 was considered statistically significant. These statistical analyses were performed using the StatView 5.0 for Windows program (SAS Institute Inc., Cary, NC).

**Results and Discussion**

Thirty micrograms lecithin, 30 μg SM or 30 μg lyssolecithin, was treated with 164 units PLase D from S. chromofuscus, and lipids were extracted. None of these phospholipids in the extracted lipids could be detected by TLC. The choline-phospholipids in crude lipids (containing 20 μg phospholipid phosphorus) extracted from dietary samples were also completely hydrolyzed by PLase D (data not shown). These results were in agreement with those reported by Gurantz et al. (12).

We were able to measure the content of the choline-phospholipids by measuring the dark-brown precipitates of the choline periodate complex spectrophotometrically (PLase D-triiodide method). The choline content of the three authentic choline-phospholipids measured by the PLase D-triiodide method was almost equal to the starting concentrations of phospholipid-phosphorus (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Choline (mmol/d)</th>
<th>phospholipids (mmol/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.585±0.037</td>
<td>6.2</td>
</tr>
<tr>
<td>2</td>
<td>0.807±0.043</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>0.938±0.031</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>1.057±0.040</td>
<td>3.8</td>
</tr>
<tr>
<td>5</td>
<td>1.141±0.076</td>
<td>6.6</td>
</tr>
<tr>
<td>6</td>
<td>1.385±0.055</td>
<td>3.9</td>
</tr>
<tr>
<td>7</td>
<td>1.573±0.052</td>
<td>3.3</td>
</tr>
<tr>
<td>8</td>
<td>1.671±0.060</td>
<td>3.6</td>
</tr>
<tr>
<td>9</td>
<td>2.179±0.079</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>2.565±0.111</td>
<td>4.3</td>
</tr>
<tr>
<td>11</td>
<td>2.780±0.080</td>
<td>2.9</td>
</tr>
<tr>
<td>12</td>
<td>2.792±0.094</td>
<td>3.4</td>
</tr>
</tbody>
</table>

The samples were the crude lipid extracted from Japanese dietary samples (6). The phospholipid contents were measured according to the methods described in the text. The data represented are the mean ± SD. Each sample was measured in triplicate. Coefficient of variation (%).

Then, the daily intake of the total choline-phospholipid content of the Japanese diet was measured by two methods, namely the PLase D-triiodide and TLC methods (Table 2). Since the coefficient of variation (CV%) in the two methods was less than 10%, the measure of the dispersion was not different between the two methods. On the other hand, the regression equation between the TLC (x) and PLase D-triiodide (y) methods was y = 0.737 ± 0.033 (r = 0.996, p < 0.05) from the mean values in Table 2. The 95% CI of the slope and intercept were 0.724 to 0.752 and –0.08 to 0.146, respectively.

After TLC separation, lecithin and other choline-phospholipids (SM and lyssolecithin) were measured individually. The choline-phospholipid contents of the diets consisted of 82.3 ± 7.4% (n = 12) lecithin. In most foods, the SM content is 20% or less than the lecithin content (13), and the lyssolecithin content is minimal due to the enzymatic degradation of lecithin.

TLC requires the use of strong acids and oxidants (7, 9). Therefore, measurement of many samples can be detrimental to the researcher’s health. The method is also not economical due to the large volume of organic solvents and many TLC plates required. Different HPLC methods can be used to determine phospholipid content, including an HPLC-electrospray ionization-isotope dilution method (14), a method for using light-scattering (15) and a method for using refractive index detection for the detector (16). It is not economical to use these methods for large-scale choline-phospholipid measurements. Hence, a more economical method
needed to be developed. Until now, an enzymatic method combining PLase D, choline oxidase and peroxidase was the most convenient method for measuring choline-phospholipids (5, 12). However, when using our new method, which does not use choline oxidase or peroxidase, the reaction procedure is simpler than that of the previous enzymatic method.

Our novel method is also one of the most convenient compared to the other methods, and is easy, economical, and suitable when measuring many samples in single experiments.

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REFERENCES