Microdetermination of Cholesterol and Phospholipid in Cerebrospinal Fluid and Serum by Silicic Acid Column Chromatography

Yung S. Shin* and James C. Lee

A method is presented for the determination of cholesterol and phospholipid, which requires 5 μl of human serum or 1-2 ml of cerebrospinal fluids. With this method 5-100 μg of cholesterol and phospholipid can be separated by a modified silicic acid column after elution of the mixture with 1 ml of chloroform and 3 ml of methanol. Recovery for 24.6 μg of cholesterol and 30.5 μg of phospholipid was 98.4 and 96.7%, respectively. Standard deviations of ±1.73 and ±1.24 have been obtained for the reproducibility of cholesterol and phospholipid determinations after chromatography. The method has been applied for the estimation of the cholesterol/phospholipid ratio and of lipid phosphorus in total phosphorus of human cerebrospinal fluids.

Though procedures for phospholipid and cholesterol assay, by means of solubility differential (1, 2) or absorption chromatography (2-4), have been available, a quantitative separation and determination of phospholipid and cholesterol from cerebrospinal fluids or extremely small amounts of serum was believed to be difficult. This was mainly due to lack of adequate micromethods for the separation of phospholipids from the other lipid substances, or for an accurate extraction of phospholipids from human cerebrospinal fluid, since other phosphorus-containing compounds are present in concentrations approximately 60 times greater than that for lipid phosphorus (5).

The procedure described below is a simple and rapid one for sepa-

From the Biochemistry Department, St. Anthony Hospital, Terre Haute, Ind.
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*Present address: Biochemistry Laboratory, St. Mary Mercy Hospital, Gary, Ind.
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ration of cholesterol and phospholipid, with simultaneous removal of nonlipid phosphorus contamination through a silicic acid column. The micromethod is based on the extraction procedure of Folch et al. (6). The separation by micro-silicic acid column chromatography is a modification of the method of Borgström (2) and Nelson and Freedman (3), with the standardization of silicic acid of Hernandez et al. (7), the colorimetric determination of cholesterol of Shin and Lee (8), and determination of lipid phosphorus of Bartlett (9).

**Materials and Methods**

**Equipment**

1. Ultramicro pipets (5, 10, 50, 100, 300 and 400 μl.)
2. Glass-stoppered centrifuge tubes (15 ml.)
3. Pyrex ignition tube (15 x 125 mm., soaked in 50% nitric acid overnight, rinsed with distilled, deionized water 5 times, and dried in an oven)
5. Nitrogen tank, with water bath set for 55–60°C
6. Oven set for 160°C
7. Beckman Model B spectrophotometer and cuvets (40 x 10 x 3 mm., (Pyrocell Manufacturing Co.) or Coleman Jr. Spectrophotometer and cuvets (12 x 70 mm., with micro-space cuvette adapter, Coleman 6-319).

**Reagents**

- **Methanol** Absolute, reagent grade
- **Chloroform** Washed twice with 1 N hydrochloric acid and twice with distilled water, filtered, dried over anhydrous sodium sulfate, and distilled (with 0.5% ethanol (v/v) added as a preservative).
- **Chloroform-methanol** 2:1, v/v
- **Glacial acetic acid** 99-100%, analytical grade (E. Merck A. G., Darmstadt)
- **FeCl₃·6H₂O color reagent** Four milliliters of the stock solution (2.5% in 87% phosphoric acid) is diluted to 50 ml. with concentrated sulfuric acid
- **Cholesterol** Fisher certified, melting point 149-150°C
- **Sulfuric acid** 10 N
- **Ammonium molybdate solution** 5% in distilled, deionized water
- **Fisk and SubbaRow reagent** 0.5 gm. of purified 1-amino-2-naph-
thol-4-sulfonic acid is added to 200 ml. of freshly prepared 15% sodium bisulfite with mechanical stirring, followed by 1.0 gm. of anhydrous sodium sulfite. The solution is filtered, stored in a dark bottle, and prepared fresh weekly.

**Preparation of Silicic Acid Column**

In preparation of the column, 50 gm. of silicic acid (Malinckrodt, AR, 100-200 mesh USP) is slurried with 150 ml. of 5N HCl in 95% ethanol (1:10, v/v) and filtered under vacuum. The procedure is repeated twice and followed by drying at 120° with intermittent stirring. The acid is next washed three times with petroleum ether in the fashion described above. This is followed by drying for 15 min. at 120°. Then it is washed three times with methanol and finally dried for 2 hours at 120°, with intermittent stirring. The prepared absorbent is activated by heating at 110-120° for 18 hours and drying in a double dessicator for at least 48 hours. A glass column or 2-ml pipet (Pyrex, 7064) which has an inside diameter of 3 mm, and is 45 cm. long, is used, with the tip of the pipet filed off just beyond the place where the pointed end begins to constrict. A fine glass-cotton plug is placed at the constricted area. A piece of polyethylene tube (0.5 cm. i.d.) is connected with the top end of the pipet or glass column, and a tube connector (make-break connector, female; W. A. Baum Co. Inc., Copiague, Long Island, N. Y.) is attached to it. Then, 160 mg. of activated silicic acid is added to the column through a capillary pipet. While suction is applied from the lower tip, the wall of the tube is tapped with a glass rod to shake down the absorbent evenly and to pack the column. The even filling and packing of the column is of the utmost importance.

**Procedure**

The spinal fluid is centrifuged and 2.0 ml. of the clear fluid is added to a glass-stoppered centrifuge tube. For serum specimens 5 μl. of the sample and 2 ml. of distilled water are mixed well in a glass-stoppered centrifuge tube. Then, 4 ml. of chloroform-methanol solution is added, mixed with a mixer for 1 min., and placed in the 55° water bath for 5 min. The tube is removed from the water bath and the contents mixed for 1 min. The heating and mixing are repeated twice and the tube is then centrifuged for 10 min. at 2500 rpm. The upper phase is carefully removed with a capillary pipet and 0.3 ml. of
methanol is added to incorporate the remaining water. The contents of the centrifuge tubes (chlooroform layer) are filtered through 7-cm. diameter Sharkskin fat-free filter paper (Schleicher and Schuell) into a plain 15-ml. centrifuge tube. The original tube as well as the filter paper are rinsed with 1 ml. of chloroform-methanol solution which is added to the previous filtrate. The filtrate is taken to dryness under nitrogen at maximum temperature of 60°.

Column Chromatography

After 0.5 ml. of chloroform is added to the dried lipid extract this is mixed with the mixer for a few seconds. Then 2 ml. of chloroform is added to the column and the tube connector and connector attached to the nitrogen tank are connected and nitrogen is forced through the column to rinse the silicic acid. After the addition of chloroform to the column the absorbent should be clear and colorless. As soon as the solvent level reaches a point just above the level of absorbent, the tube connectors are disconnected. The lipid extract, in 0.5 ml. of chloroform, is transferred to the column using a capillary pipet with a very sharp constricted end. A cuvet is placed under the column and the solvent is forced through the column. The flow rate should be about 1 drop per second and never greater than 3 ml. per minute. Care should be taken never to allow the solvent to go below the top level of absorbent. The tube is rinsed with two 0.5-ml. portions of chloroform, which are passed through the column as described above. Next, 3 ml. of methanol is added and passed through the column. When the methanol begins to flow through, a white opaque layer appears in the absorbent. The chloroform is collected until the white layer moves down to the end of the column. The tube adapter is then disconnected and the tip of the column rinsed with a few drops of chloroform. The methanol fraction is then collected into a Pyrex ignition tube and, after collection, the tip of the column is rinsed with few drops of chloroform. Solvent blanks are prepared and used as blanks. The solvents are taken to dryness under nitrogen at a maximum temperature of 60°.

Colorimetric Determination

Cholesterol

To the dried lipid extract from the chloroform tube 400 μl. of glacial acetate and 350 μl. of ferric chloride-sulfuric acid color reagent are added using an ultramicro pipet. The contents are mixed with the
miller for few seconds. The cuvets are rotated gently by hand to wash
down the walls and are then placed in a boiling-water bath for exactly
1 min. The cuvets are removed and cooled in a beaker containing
cold water. The color is read at 530 m or with the Coleman Jr. spectro-
photometer, utilizing the micro-space adapter, as the contents are
transferred into a 10 x 3 x 40-mm. micro cuvet (Pyrocell Manufactur-
ing Co.) and read at 530 m with a Beckman B spectrophotometer.
Then, 1 ml of the standard solutions, containing 5–20 µg of choles-
terol are evaporated to dryness and determined as previously de-
scribed, for a standard curve.

Phospholipid

To the dried lipid extract in the Pyrex ignition tube, 0.3 ml of 10N
sulfuric acid is added and placed in the oven for 1½ hours at 160°.
After 2 drops of 30% hydrogen peroxide are added to the solution the
tubes are returned to the oven for at least 1½ hours or more to com-
plete the combustion and to decompose all the peroxide. When the
tubes are cool 650 µl of distilled, deionized water are added and 200
µl of 5% ammonium molybdate solution, using ultramicro pipet.
After mixing, 50 µl of Fisk SubbaRow reagent is added and the tube
contents again mixed well with the mixer. The tubes are covered with
glass marbles and placed in the boiling-water bath for exactly 7 min.
The contents are cooled and transferred into 40 x 3 x 10-mm. cuvets
and the color is read at 830 m with the Beckman Model B spectro-
photometer equipped with a red-sensitive phototube, as the contents
are transferred to 12 x 70-mm. cuvets equipped with micro-space
adaptors and the color read at 700 m with a Coleman Jr. spectro-
photometer.

For a standard stock solution, 351 mg of pure dry monopotassium
phosphate is dissolved in 10 ml of 10N sulfuric acid, diluted to 1 L.
For the marking standard, 10 ml of the stock solution is diluted to
100 ml with water. The resulting solution contains 0.8 µg of phos-
phorus in 100 µl. Standard solutions containing from 0.4 µg to 2.0 µg
of phosphorus are used for the standard curve. The phospholipid
concentration is calculated from the amount of lipid phosphorus
multiplied by a factor of 25.

Results and Discussion

Procedures for the microdetermination of phospholipids utilizing
differential solubility are subject to criticisms (10–12) because the
methods are based on solubility characteristics of complex substances, which differ markedly from their solubilities in the pure state \((13, 14)\). Application of absorption chromatography using magnesium oxide \((2)\), magnesia \((4, 15)\), or alumina columns \((16, 17)\) for the isolation of phospholipid from cholesterol or other lipids have been reported. Recently it has been shown that these could be replaced by silicic acid, since the latter is considered a true inert absorbant \((18, 19)\). The quantitative separation of phospholipid from cholesterol or other lipids using silicic acid has been made by Borgström \((2)\), and advanced by Nelson and Freedman \((3)\), and, later, by Lea and Rhodes \((20)\). These methods required a large amount of serum \((10 \text{ ml.})\) and eluents, and their application to microchemistry has been limited.

An effort has been made so that one may be able to apply a smaller amount of sample for lipid fractionation through the column. Such a column has been prepared by enhancing the activity of the absorbent. Studies have been made on the recovery of known quantities of material through various sizes of columns, using various amounts of absorbent and solvents. Recovery on the proposed column is shown in Table 1. Throughout this study, known standard cholesterol solutions and purified phospholipids from human serum have been used. For the preparation of the phospholipids, 20 ml. of pooled human serum was used and phospholipids precipitated by acetone and saturated magnesium chloride in ethanol \((12)\). The phospholipids are re-extracted from the precipitate and purified by the method of Nelson and Freedman \((3)\). It has been indicated that the column permits considerably wider range of cholesterol and phospholipid \((5-100 \mu g.)\). An advantage of this column over the micromethod using differential solubility in solvents is that the sample passed through the column is free of nonlipid phosphorus, whereas, with some of the solvent pro-

<table>
<thead>
<tr>
<th>Table 1. Recovery of Cholesterol and Phospholipids after Column Chromatography</th>
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</thead>
<tbody>
<tr>
<td>Before chromatography</td>
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<tr>
<td>Cholesterol ((\mu g.))</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>10.0</td>
</tr>
<tr>
<td>25.0</td>
</tr>
<tr>
<td>50.0</td>
</tr>
<tr>
<td>100.0</td>
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</table>
procedures, contamination has been encountered. Determination of phosphorus on chloroform fraction of the present procedure was made, and phosphorus in this fraction was undetectable. Also, cholesterol was determined on the methanol fraction and was found to be 1.8 to 5% when the concentration exceeded 25 \( \mu g \). Reproducibility of the column chromatography has been determined (Table 2). Standard deviation of \( \pm 1.73 \) and \( \pm 1.24 \) have been obtained for the determination of cholesterol and phospholipid respectively, after chromatography, while the colorimetric determination of cholesterol and phospholipid without passing through the column gives standard deviation of 4.28% and 1.75%. The method was applied to a series of spinal fluids of hospitalized individuals (Table 3). It can be used for

### Table 2. Reproducibility of Column Chromatography

<table>
<thead>
<tr>
<th>No. of determination</th>
<th>Cholesterol (( \mu g. ))</th>
<th>Deviation</th>
<th>Phospholipid (( \mu g. ))</th>
<th>Deviation</th>
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<tbody>
<tr>
<td>1</td>
<td>24.4</td>
<td>0.0</td>
<td>30.5</td>
<td>0.1</td>
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<tr>
<td>2</td>
<td>24.8</td>
<td>0.4</td>
<td>32.0</td>
<td>1.4</td>
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<tr>
<td>3</td>
<td>22.0</td>
<td>2.4</td>
<td>28.9</td>
<td>1.7</td>
</tr>
<tr>
<td>4</td>
<td>23.6</td>
<td>0.8</td>
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<tr>
<td>5</td>
<td>27.1</td>
<td>2.7</td>
<td>30.0</td>
<td>0.6</td>
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<tr>
<td>6</td>
<td>25.5</td>
<td>1.1</td>
<td>29.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Average</td>
<td>24.4</td>
<td>( \pm 1.73 )</td>
<td>30.6</td>
<td>( \pm 1.24 )</td>
</tr>
<tr>
<td>S. D.</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### Table 3. Cholesterol, Phospholipid, and Total Phosphorus in Cerebrospinal Fluid of Hospitalized Individuals

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cholesterol (mg./100 ml.)</th>
<th>Lipid phosphorus (mg./100 ml.)</th>
<th>Phospholipid (mg./100 ml.)</th>
<th>Total phosphorus (mg./100 ml.)</th>
<th>Cholesterol/phospholipid ratio</th>
<th>Lipid phosphorus in total phosphorus (%)</th>
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<tbody>
<tr>
<td>RM</td>
<td>.535</td>
<td>.0173</td>
<td>.433</td>
<td>1.20</td>
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<td>.322</td>
<td>.0201</td>
<td>.502</td>
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<td>.66</td>
<td>1.5</td>
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<tr>
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<td>.0270</td>
<td>.676</td>
<td>1.46</td>
<td>.80</td>
<td>1.7</td>
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<tr>
<td>BG</td>
<td>.400</td>
<td>.0300</td>
<td>.750</td>
<td>1.95</td>
<td>.53</td>
<td>1.5</td>
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<tr>
<td>SD</td>
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<td>.0960</td>
<td>2.400</td>
<td>1.80</td>
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<td>.80</td>
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</tr>
<tr>
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<td>.525</td>
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<tr>
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<td>1.48</td>
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<td>6.1</td>
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<tr>
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<td>.502</td>
<td>1.58</td>
<td>1.08</td>
<td>1.2</td>
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<tr>
<td>KB</td>
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<td>.0201</td>
<td>.502</td>
<td>1.76</td>
<td>1.49</td>
<td>1.2</td>
</tr>
<tr>
<td>EM</td>
<td>.500</td>
<td>.0248</td>
<td>.620</td>
<td>1.32</td>
<td>.81</td>
<td>1.9</td>
</tr>
<tr>
<td>HD</td>
<td>.310</td>
<td>.0135</td>
<td>.338</td>
<td>1.10</td>
<td>.92</td>
<td>1.5</td>
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routine clinical microanalysis, and many samples may be determined simultaneously. Determinations in cerebrospinal fluids of normal subjects and patients with various disorders and correlation with pathology will be continued and discussion in detail will appear in another publication.

References