Densitometry of Phosphatidylcholine and Sphingomyelin in High-Density Lipoproteins

Gerd Schmitz, Hans-Ulrich Jabs, and Gerd Assmann

We describe the quantitative densitometric determination of phosphatidylcholine (PC) and sphingomyelin (SP) in human serum after precipitation with phosphotungstic acid/MgCl₂ and use of thin-layer chromatography. After development, chromatographic plates were charred with methanolic sulfuric acid and MnCl₂ and scanned by direct reflectance densitometry in an automated densitometric system interfaced to a basic programmable computing integrator. The method is sensitive enough to detect abnormally low concentrations of PC and SP in high-density lipoproteins. The accuracy of the method was tested either with the Bartlett phosphorus assay or with enzymatic methods for PC and SP; correlations of the described method with the enzymatic determinations were r = 0.93 and 0.88, respectively. Day-to-day precision (CV) for the phospholipid determination was 8.6% for PC and 12.2% for SP. The major advantage of this inexpensive technique is that native plasma or serum or the serum supernatant after precipitation can be used without prior delipidation. With this technique serum high-density lipoproteins had PC values of 1.08 (SD 0.32) mmol/L in men (n = 158) and 1.12 (SD 0.37) mmol/L in women (n = 192); similarly, SP values were 0.23 (SD 0.07) mmol/L in the men and 0.23 (SD 0.08) mmol/L in the women. The differences by sex are not significant.

Additional Keyphrases: precipitation with phosphotungstic acid/MgCl₂ • reference interval • noncholesterol content of high-density lipoproteins • thin-layer chromatography

Recent clinical and epidemiological studies (1, 2) reveal a strong negative correlation between the risk of coronary heart disease and concentrations of high-density lipoprotein (HDL). Generally, HDL is measured as cholesterol in the supernate of serum or plasma after other lipoproteins have been precipitated with polyanions (2). However, the HDL cholesterol concentration does not represent the total HDL mass, and it has recently been demonstrated (3–6) that analysis for HDL-apolipoprotein A-I may yield different results concerning the risk of coronary heart disease. HDL phospholipids have been analyzed only in a few studies, by enzymatic methods that allow the determination of cholesterol-containing phospholipids (7, 8). The major disadvantage of these methods is that the supernate remaining after PTA/MgCl₂ precipitation contains considerable amounts of lyso-phosphatidylcholine. Recently, new enzymatic methods have been developed by which PC (9) and SP (10) in the PTA/MgCl₂ supernate can be determined specifically. Now we describe a method for the quantitative densitometric determination of PC and SP in whole plasma or serum or in the PTA/MgCl₂ supernate from human serum; its precision is comparable with that for the enzymatic methods.

Materials and Methods

Reagents

All organic solvents were of LiChrosolv® quality (Merck, Darmstadt, F.R.G.). "High-performance" thin-layer chromatographic (HPTLC) plates (10 × 20 cm, cat. no. 5642; Merck) were obtained for the phospholipid separation. To avoid oxidation, we added butylated hydroxytoluene (Fluka; no. 34750) to all organic solvents at 0.5 mL/L. The charring reagent consisted of 3.2 g of MnCl₂, 32 mL of concentrated sulfuric acid, 4800 mL of methanol, and 480 mL of deionized water. The PTA/MgCl₂ reagent was from Boehringer Mannheim (no. 400971).

Procedures

Precipitation with phosphotungstic acid/magnesium chloride. To precipitate the apolipoprotein B-containing lipoproteins, mix 200 μL of serum or plasma with 20 μL of the PTA/MgCl₂ reagent. Further details are to be published separately. After 10 min at room temperature, centrifuge the samples (for 2 min, 12 000 × g). We used a Hettich microscale centrifuge. Separate the supernate from the precipitate and, without delay, apply the supernate to the HPTLC plate.

Sample application, chromatography, and detection. To apply native serum, plasma, or PTA/MgCl₂ supernatant to the HPTLC plates, we used the Camag-Nanomat pipettor (Camag, Muttenz, Switzerland) with disposable 0.5-μL capillaries. The plates were developed for 10 min in the Camag linear development chamber (cat. no. 28520) with 4 mL of chloroform/methanol/water (65:25:4 by vol) to which butylated hydroxytoluene had been added as mentioned. The plates were then dried for 10 min in an oven at 110 °C, immersed in a chromatography tank with the charring reagent for 20 s, and finally rested horizontally on a paper towel to remove excess reagent from the back. We then reheated the plates for 30 min at 110 °C in the oven, placing each one on a Teflon block to ensure even heating (ms. submitted for publication).

Calibration. To calculate the concentration of the phospholipids detected, all HPTLC-plates have five-point calibrators for PC and SP. For standards, we used either native pooled serum or serum diluted with isotonic saline (two-, three-, four-, five-, and ninefold). The standards were stored at −70 °C in 50-μL aliquots. After chromatographic separation we determined the phospholipid content of the serum pool, both enzymatically (9, 10) and chemically with the phosphorus assay according to Bartlett (11). To evaluate accuracy, we added PC and SP standards to pooled serum and measured with the enzymatic and chemical technique to eliminate matrix effects. Accuracy and day-to-day preci-

Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, Demagkstrasse 3, 4400 Münster, F.R.G., and Institut für Arteriosklerosforschung an der Universität Münster.

1 Nonstandard abbreviations: HDL, high-density lipoproteins; HPTLC, "high-performance" thin-layer chromatography; PC, phosphatidylcholine; PTA, phosphotungstic acid; and SP, sphingomyelin.

Received Dec. 24, 1982; accepted April 13, 1983.
tion were also determined with thawed samples from a
frozen serum pool for each HPTLC plate.

Quantification. We quantified the separated phospholipids as described elsewhere (ms. submitted for publication) with a fully automated Camag TLC-Scanner interfaced to a SP 4100 basic integrator (Spectra Physics, Darmstadt, F.R.G.) and a Kerr-minifile 4100 D (Spectra Physics) for data storage. The basic program developed for this method is described in detail elsewhere (ms. submitted for publication).

Results and Discussion

To separate PC and SP in the PTA/MgCl2 supernate, we used HPTLC with the classical solvent system chloroform/methanol/water (65/25/4, by vol); as Figure 1 shows, PC and SP are clearly separated. The dark spot at the origin contains proteins, whereas neutral lipids migrate with the solvent front. We tested other separation systems, but the best results for delipidation, separation of the spots, and optimal spot background conditions were obtained with the solvent system specified. With this method the background/peak ratio is adequate even for low concentrations of phospholipids and yields satisfactory coefficients of variation of the integrated peak areas.

Standard curves for PC and SP derived from serum diluted with isotonic saline are shown in Figure 2. PC can be quantified with the described method between 0.12 and 1.70 mmol/L and SP between 0.02 and 0.28 mmol/L. Standard curves prepared from different concentrations of PC and SP dissolved in chloroform have slightly different slopes from those prepared from serum standards—0.74 vs 0.79 for PC and 0.83 vs 0.75 for SP—owing to the different spot geometry of the phospholipids in the different matrices, but there was a good correlation (r = 0.98, n = 15) between both systems. We used serum-based standards for all plates, serum being easily available and less expensive than purified phospholipid compounds in organic solvents; serum also helps protect the phospholipids from oxidation and presents a matrix similar to that of the samples to be quantified. We studied the correlations between the enzymatic method, the phosphorus determination, and the densitometric method (y) for quantifying the separated phospholipids in 20 samples. For PC concentrations of about 0.5 to 1.35 mmol/L the densitometric vs enzymatic method gives a correlation of r = 0.93 (y = 0.77x + 15); for densitometry vs phosphorus determination r = 0.88 (y = 0.96x + 2.4). For SP concentrations of about 0.18 to 0.30 mmol/L, the respective correlations are r = 0.84 (y = 0.89x + 4.8) and r = 0.86 (y = 0.88x + 2).

The coefficients of variation within run for PC and SP (6.2% and 7.9%) were determined for six different HPTLC plates with 30 sample applications per plate. The day-to-day precision (CV) of the densitometric method was determined as 8.6% for PC and 12.2% for SP (n = 60 days). We evaluated the stability of PC and SP in sera stored at −70°C over a four-month period, and no differences in absolute values were detected.

With this technique we found, for 158 men, mean (±SD) concentrations of HDL-PC and HDL-SP of 1.08 ± 0.32 and 0.23 ± 0.07 mmol/L, respectively, and, for 192 women, 1.12 ± 0.37 and 0.23 ± 0.08 mmol/L, respectively (Table 1).

Using the analytical procedure for HDL-PC and HDL-SP that we describe, one can quantify these lipids directly without prior delipidation of samples or isolation of HDL by ultracentrifugation. Given the rapid improvement in the HPTLC techniques during the last years (12–15), and our own work on automating lipid microanalysis in tissues and lipoproteins, we suggest that this method is a useful addition to previously described procedures (9, 10) for the enzymatic determination of HDL-PC and HDL-SP. The method is fast, inexpensive, and suitable for automation. More than 30 samples can be quantified on each HPTLC plate, in the concentration ranges of 0.12–1.7 mmol/L for PC and 0.02–0.28 mmol/L for SP. Moreover, the densitometric procedure is less expensive than the enzymatic one.

In contrast to the well-established biochemical, clinical, and epidemiological relationship between HDL-cholesterol and the risk of coronary heart disease, the potential role of HDL-phospholipids in the prediction and early recognition of patients at risk for atherosclerotic disease is largely

<table>
<thead>
<tr>
<th>Table 1. HDL-PC and HDL-SP Concentrations in Apparently Healthy Company Employees, as Determined Densitometrically</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HDL-PC</strong></td>
</tr>
<tr>
<td><strong>Men</strong> (n = 158)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>SD</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Minimum</td>
</tr>
<tr>
<td>Maximum</td>
</tr>
<tr>
<td>Percentile, 5</td>
</tr>
<tr>
<td>Percentile, 25</td>
</tr>
<tr>
<td>Percentile, 75</td>
</tr>
<tr>
<td>Percentile, 95</td>
</tr>
</tbody>
</table>
unknown. Now that a procedure is available for quantifying HDL-phospholipids in population studies, large-scale epidemiological studies are now underway in our laboratory to determine the role of various HDL components in the prediction of coronary atherosclerotic risk.

We thank Mrs. M. Lencyk and Mr. W. Rensinghoff for excellent experimental work and technical assistance.

References

Enzyme Immunoassay of Thyroxin-Binding Globulin in Dried Blood Samples on Filter Paper
Naoshige Hata,1 Masao Ito,3 Hitoshi Mizuta,1 Osamu Nose,2 and Kiyoshi Miyal1

A double-antibody enzyme immunoassay was developed for determination of thyroxin-binding globulin in dried blood samples on filter paper. The measurable concentration range of thyroxin-binding globulin in two 3-mm blood discs was 3.3 to 52 mg/L equivalent of serum (i.e., equivalent to the concentrations in known serum standards). Thyroxin-binding globulin in dried blood samples on filter paper was stable for at least four weeks when kept dry at –20 °C, 4 °C, or room temperature. The mean coefficients of variation were 6.6% (within assay) and 5.9% (between assays). The concentrations of thyroxin-binding globulin in dried blood samples determined by this method correlated well with those in serum determined by radioimmunoassay (r = 0.95) and by enzyme immunoassay (r = 0.96). This method is applicable for detecting cases of thyroxin-binding globulin deficiency and avoids the false-positive results for neonatal hypothyroidism obtained by measuring thyroxin.

Additional Keyphrases: screening · hypothyroidism · neonates · reference intervals

Because congenital hypothyroidism, which causes irreversible mental retardation, can be prevented by early treatment (1), mass screening for this condition is carried out throughout the world. Several screening methods have been developed for this purpose, such as measurement of total thyroxin and thyrotropin concentrations in dried blood samples on filter paper (2–7). Measurement of total thyroxin is useful for detecting primary, secondary, and tertiary hypothyroidism but gives false-positive results for patients with thyroxin-binding globulin (TBG)4 deficiency (hypo-TBG), who do not need to be treated. Therefore, Dussault et al. (8, 9) measured TBG in dried blood samples on filter paper by using radiolabeled thyroxin when the total thyroxin concentration was low. However, the large scale of screening programs makes a nonspecific method preferable. Here we describe an enzyme immunoassay (ELA) for meas-

1 Central Laboratory for Clinical Investigation and Department of Laboratory Medicine, and
2 Department of Pediatrics, Osaka University Medical School, Osaka 553, Japan.
3 Eiken Immunochemical Laboratories, Tokyo 114, Japan.

Received Feb. 7, 1983; accepted April 20, 1983.

4 Nonstandard abbreviations: TBG, thyroxin-binding globulin; hypo-TBG, thyroxin-binding globulin deficiency; ELA, enzyme immunoassay; MBS, m-maleimidobenzoyl N-hydroxysuccinimide ester; BSA, bovine serum albumin.