Simple, Direct Measurement of Lipoprotein X in Serum

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This relatively simple method for quantitative estimation of lipoprotein X is based on assay of phospholipids in precipitates of lipoprotein X. We first remove other lipoproteins by precipitation with phosphotungstic acid solution. Lipoprotein X is then precipitated from the supernatant fluid with an alkaline solution of magnesium ion, and phospholipid is determined in this precipitate. Results are linearly related to concentration of lipoprotein X. The CV is <3%. Results correlated well with those for agar gel electrophoresis.

Additional Keyphrases: enzymic phospholipid assay • comparison with electrophoresis in agar gel • cholestasis assessment

In 1967, Switzer (1) separated lipoprotein X (LP-X) from sera of patients with cholestasis. Seidel et al. (2) identified lipoprotein X as the lipoprotein migrating cathodally on agar gel electrophoresis, characterized by its unique lipid composition. Lipoprotein X is determined in clinical laboratories as a reliable indicator of cholestasis (3, 4). Recently many workers have published methods for estimating lipoprotein X, but these methods (5–9) have the disadvantages of being complicated and requiring standard material and the use of organic solvent, and so they are not well suited for routine use.

Burstein and Scholnick (10) reported that a polyanion at acid pH in the absence of metal ions precipitates low-density lipoprotein, very-low-density lipoprotein, chylomicrons, abnormal lipoprotein, and high-density lipoprotein, in that order. We observed no precipitation of lipoprotein X under these conditions.

Here, we describe a method for the quantitative estimation of lipoprotein X. Low-density lipoprotein, very-low-density lipoprotein, and chylomicron are precipitated from the serum by treatment with a solution of phosphotungstic acid. An alkaline solution of magnesium ion, added to the supernate, precipitates lipoprotein X, but not high-density lipoprotein. Lipoprotein X is estimated by assay of the phospholipids in the precipitate.

Materials and Methods

Samples: Blood samples were obtained from patients with various diseases. From these samples, sera were prepared by low-speed centrifugation of the freshly collected blood. Samples containing lipoprotein X were identified from its precipitation with a polyamic compound on agar gel (11).

Electrophoresis on agar gel: For electrophoresis we used a 10 g/L solution of Bacto Agar (Difco, Detroit, MI 48232) in barbital buffer (50 mmol/L, pH 8.6). Runs were for about 1 h at 200 V. After electrophoresis, lipoprotein X was identified by mixing the plate with the polyamic compound (a 0.54 mol/L solution of MgCl2 containing 40 g of phosphotungstic acid per liter).

For immunoelectrophoresis, after electrophoresis on agar gel, antisera were added to the trough and allowed to diffuse for 16 to 24 h. Antisera to α-lipoprotein, β-lipoprotein, and lipoprotein X were from Behringwerke AG, Marburg, F.R.G. A β-lipoprotein antibody contaminating lipoprotein X antiserum was removed by treatment with β-lipoprotein on the agarose solid phase, Sepharose 4B.

Lipid analysis: Phospholipids, cholesterol, and triglycerides were determined enzymically with commercially available test kits (International Reagents Co., Kobe, Japan).

Separation and quantitative estimation of lipoprotein X: To 50 µL of sample (patient’s serum), add 50 µL of Reagent I [80 g of phosphotungstic acid and 10 mmol of EDTA per liter of 0.2 mol/L 2-(N-morpholino)ethanesulfonic acid buffer, pH 5.3]. After 10 min at 25 °C, centrifuge the mixture (10 min, 1500 g, room temperature). Pipet 50 µL of the clear supernate into a test tube, add 0.1 mL of Reagent II (20 mmol of MgCl2 per liter of 0.1 mol/L Tris HCl buffer, pH 9.0), and mix well. After 10 min, centrifuge the mixture (1500 g, 10 min, room temperature). Completely remove the supernatant fluid with a micropipette connected to an aspirator. Determine the phospholipids in the precipitate with a commercially available test kit, which involves the use of phospholipase D (EC 3.1.4.4) choline oxidase (EC 1.1.3.17), and peroxidase (EC 1.1.1.7). Calculate the concentration of lipoprotein X as follows: (absorbance of sample/absorbance of standard) × phospholipid standard concn (mg/L) × 2 × 1.5 = lipoprotein X, in mg/L.

The first precipitate mentioned above was washed with an equivolume mixture of Reagent I and isotonic saline, the second precipitate with a 1:1/4 (by vol) mixture of Reagent I, saline, and reagent II. These washed precipitates were dissolved in a 30 g/L solution of NaHCO3 and used in the analytical experiments described below.

Results

Specimens from patients with cholestasis were fractionated as described. We then electrophoresed these subfractions, (supernate I, precipitate I, supernate II, and precipitate II) on agar gel, and diluted and identified lipoprotein X by use of the polyamic compounds. As shown in Figure 1, lipoprotein X was present in supernate I and precipitate II on the cathodal side of agar gel, but not in either precipitate I or supernate II. Clearly, all lipoprotein X in the native serum was recovered in precipitate II.

To obtain further information on the quality of the separation of lipoprotein X and other lipoproteins, we analyzed native serum and its subfractions by immunoelectrophoresis on agar gel (Figure 2). Supernate I and precipitate II showed a precipitin line with lipoprotein X on the cathodal side of the well, but this was not the case for precipitate I and supernate II. β-Lipoprotein was identified in precipitate I, and α-lipoprotein in supernate II. From these pieces of evidence it is clear that lipoprotein X was separated in precipitate II from other lipoproteins in serum.

Precipitate II was analyzed for its lipid composition. The results: 63% (SD 2.5%) phospholipids, 33% (SD 2.1%) free

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cholesterol, 2% (SD 1.5%) esterified cholesterol, and 2% (SD 1.2%) triglycerides. These results are similar to those of Seidel et al. (2).

We evaluated the present method in three ways, as follows:

Lipoprotein X-positive serum was diluted with normal serum, and the lipoprotein concentrations in these diluted samples were measured by the present method. As shown in Figure 3, lipoprotein X concentration and dilution ratio were linearly related.

**Discussion**

Lipoprotein X is present in the serum of patients with cholestasis, and Gerson et al. (13) pointed out that it is a reliable indicator of cholestasis, more so than alkaline phosphatase. Ritland (14) noted that lipoprotein X concentrations in serum in patients with extrahepatic cholestasis exceeded those in patients with intrahepatic cholestasis. In contrast, Simon and Poon (15) suggest that serum lipoprotein X quantitation has little or no clinical value in distinguishing extrahepatic from intrahepatic cholestasis. In spite of the importance (real or potential) of lipoprotein X measurement, no easy method for lipoprotein X quantitation in clinical laboratories is available. The best method hitherto, agar gel electrophoresis, is limited to lipoprotein X migrating to the cathodal side, and immunoelectrophoresis is interfered with by other lipoproteins containing Apo C.

In 1972, Burstein and Scholnick (10) noted that an acid solution of polyamion precipitates low-density lipoprotein, very-low-density lipoprotein, chylomicrons, abnormal lipoproteins, and high density lipoproteins, in that order. This suggested the present procedure. In consideration of the variation in phospholipid content of lipoprotein X in various forms of diseases, we suggest use of the factor calculated from both phospholipid and cholesterol content in lipoprotein X. In immunoelectrophoresis, we suggest that lipoprotein X, like the precipitin line observed in precipitate I, may be a \( \beta_2 \)-lipoprotein (Figure 2).

This present method is suitable for the estimation of lipoprotein X in the clinical laboratory and will be useful for studying its clinical significance in various diseases.

**References**

1. Switzer, S., Plasma lipoproteins in liver disease: I. Immunologically