

## Direct Method for Measuring Lipoprotein-X in Serum

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We describe a fast and easy method for routine quantitation of the abnormal lipoprotein, lipoprotein-X. The procedure is based on densitometry of precipitation areas obtained for it after serum electrophoresis in agar gel followed by precipitation with polyanions. The coefficient of variation was less than 3% in one series. Results were linearly related to concentration in the range 0.063 to 6.3 g/liter.

**Additional Keyphrases:** *cholestasis* • *lipoprotein-X* • *"kit" method* • *diagnostic aids*

In recent years it has been well documented that the characteristic plasma lipid changes accompanying cholestasis are primarily the result of the presence of an abnormal low-density lipoprotein (1-5) that is generally designated "LP-X."

The composition of the isolated lipoprotein is unique (3): 6% protein, 66% phospholipids, 22% unesterified cholesterol, 3% cholesterol esters, and 3% triglycerides. One important characteristic feature of LP-X is its mobility toward the cathode on agar-gel electrophoresis (3). This phenomenon, the basis of most of the LP-X determinations described so far, is not the result of a positive charge on the particle at pH 8.6, but of a pronounced electro-endosmosis in this medium, which strongly affects the migration of this rather large particle [ $70$  to  $80 \times 10^{-9}$  m (6, 7)] with its relatively small protein component.

The introduction of an immunological test for LP-X (8) added a new and simple analytical method for diagnosis of cholestasis. Several studies of adults and children (9-15) have consistently shown the specificity of qualitative demonstration of LP-X for the diagnosis of cholestasis. Apart from patients suffering from extra- or intrahepatic cholestasis, LP-X was found only in the rare inherited disease of lecithin:cholesterol acyl transferase (EC 2.3.1.43) deficiency (16, 17). LP-X could also be demonstrated in various animal species in which cholestasis had been experimentally induced (18, 19).

More recently, various workers have attempted to estimate the concentration of LP-X (8, 20-22) in order to observe possible fluctuations during the course of a disease and to study in more detail the metabolism of this abnormal plasma lipoprotein fraction.

However, all these techniques have their disadvantages for routine work: they are time-consuming and laborious, require specific antisera (8, 21), or they are based on indirect determinations by estimating various lipid constituents of the particle (20, 22).

Here, we describe a simple and direct technique for LP-X quantitation by means of a commercially available test kit.

### Material and Methods

**Samples:** Blood was sampled from patients with obstructive jaundice whose plasma contained the abnormal lipoprotein LP-X. The subjects had fasted overnight. The serum was promptly separated by centrifugation ( $4000 \times g$ ) and either stored at  $4^\circ\text{C}$  before analysis or used for isolation of LP-X.

**Isolation of LP-X:** Because isolated LP-X is less stable than LP-X in whole serum, and is not commercially available, freshly isolated preparations were used to establish the standard curve. Pure and intact LP-X was isolated individually from six different patients (five cases of extrahepatic obstruction, one of intrahepatic obstruction). The serum was first ultracentrifuged for 24 h at  $250\,000 \times g$ , at a hydrated density of 1.035 g/ml and  $4^\circ\text{C}$ , with a type 60 Ti rotor and the Heraeus Christ (D-3360 Osterode, G.F.R.) ultracentrifuge, type Vacufuge. The bottom fraction containing LP-X was separated by a tubelisting technique and further fractionated by cold ethanol precipitation (Cohn fractionation) as earlier described in detail (3). The filtrate (corresponding to Cohn fractions IV-VI) was again ultracentrifuged [ $350\,000 \times g$ , rotor type 65 Ti, Omega II Ultracentrifuge (Heraeus Christ)] at a hydrated density of 1.063 g/ml. The top fraction contained only LP-X in concentrated form. Such fractions were dialyzed vs. distilled water at  $4^\circ\text{C}$  for 48 h and then three 1-ml ali-

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quots were lyophilized and weighed on an electromicrobalance to determine the exact concentration of LP-X in the preparation. Human albumin (Behring-Werke, D-3550 Marburg/Lahn, G.F.R.) in a final concentration of 50 g/liter was then added to the isolated fraction to preserve the stability of LP-X and so that experimental conditions would be comparable to those for whole serum.

**Electrophoresis:** To samples of whole serum from healthy volunteers, a well-defined amount of isolated LP-X was added, and 10- $\mu$ l volumes were agar-electrophoresed with the "Rapidophor all in for LP-X" system (Immuno AG, Industriestrasse, A-1010 Vienna, Austria) (23) for 30 min. The LP-X band was then made visible by layering the plates for 30 min with the polyanionic compounds of the Rapidophor test kit.

**Immunology:** Immunoelectrophoresis and double immunodiffusion were performed in Difco-Bacto agar (Difco, Detroit, Mich. 48232), 10 g/liter, as detailed earlier (4) with use of specific antisera produced in this laboratory.

**Quantitation of LP-X:** Samples (volume 10  $\mu$ l) were agar-electrophoresed with the Rapidophor system for 30 min and subsequently incubated for 30 min with the polyanionic compounds of the test kit. The agar plate was then sliced and the slices transferred to a glass plate (16  $\times$  26  $\times$  1 mm) and submitted to densitometry. For each sample the total precipitin area of LP-X was measured (Figure 1). The conditions we used for densitometry were:

Instrument:	Integraph (Bender and Hobein, Zürich, Switzerland)
Standardization:	Adjustment to 100
wavelength:	500 nm
filter:	E. 0.25 (Type N G 11, Schott, Mainz, G.F.R.)
diaphragm:	2.5
amplification:	1
distance:	4 cm
Measurement:	
wavelength:	500 nm
diaphragm:	5
amplification:	6
maximum sensitivity setting	

From the standard curve, prepared as described here with isolated LP-X, the LP-X concentration is calculated as follows: (Absorbance  $\times$  0.1) - 0.007 = LP-X, in g/liter.

The densitometer we used (Integraph CH) can automatically scan electrophoretic and other separations. It measures photoelectrically the absorbance of electrophoretic separations and records a linear extinction curve with the aid of a logarithmic amplifier. The area of a fraction is determined automatically and the value indicated in digital terms (linear extinction) as percentage of the standard setting (100).

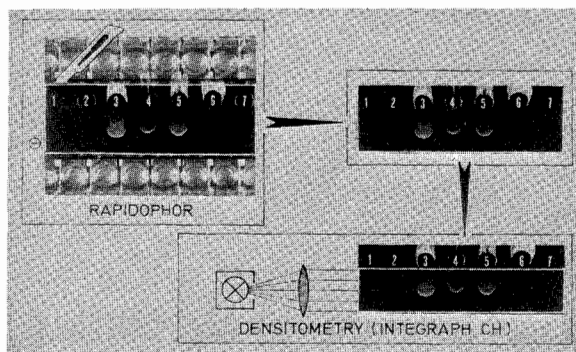


Fig. 1. Procedure for quantitating LP-X

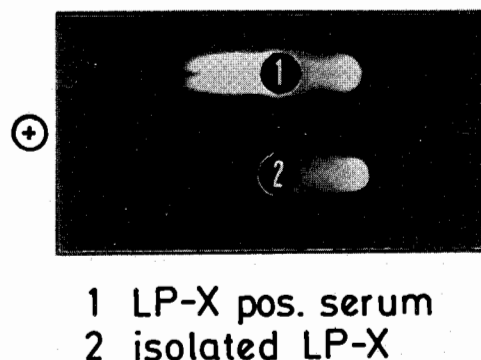


Fig. 2. Quality of isolated LP-X

Electrophoresis in agar gel (10 g/liter). The lipoprotein fractions were made visible by layering the plate with a solution of  $MgCl_2$  (0.1 mol/liter) containing sodium heparin (1.5 g/liter; 250 000 units/liter) and NaCl (13 g/liter). 1, whole serum (LP-X positive); 2, isolated LP-X from the same serum (1)

## Results

As indicated (Figure 2), the isolated LP-X fraction used as reference to establish the standard curve had an electrophoretic mobility and precipitin behavior identical to that of LP-X in whole serum. Immunologically, it developed a precipitin line only with antibodies to LP-X (Figure 3).

Each experimental point of the standard curve (Figure 4) was obtained with LP-X preparations of six different patients. The curve showed a linear response from 63 mg to 6.3 g of LP-X per liter, with a CV of less than 5%.

LP-X concentrations in sera from patients with cholestasis are generally within this range. However, at the outset of a disease concentrations may be less than 60 mg/liter, while in severe cases concentrations exceeding 6 g/liter are possible. In the latter cases, dilution of this serum with an equal volume of LP-X-negative serum will provide a basis for exact quantitation. With the Rapidophor system it is possible qualitatively to detect LP-X concentrations down to 20 mg/liter but exact quantitation is only possible above concentrations of 60 mg/liter.

The coefficient of variation ( $n = 20$ ) for this technique was less than 3% in one series and less than 5% from day to day over a period of 20 days.

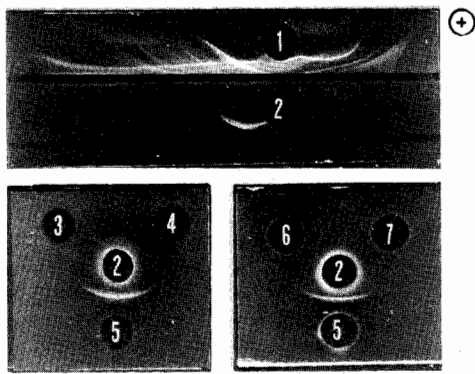


Fig. 3. Purity of isolated LP-X as indicated by immunoelectrophoresis and immunodiffusion in agar-gel (10 g/liter)

1. Whole serum (LP-X negative); 2. Isolated LP-X; 3. Anti-β-LP serum (rabbit); 4. Anti-α-LP serum (rabbit); 5. Anti-LP-X serum (rabbit); 6. Anti-human albumin serum (rabbit); 7. Anti-human globulin serum (rabbit). In the immunoelectrophoresis the upper trough contains anti-human serum; the lower trough contains anti-LP-X serum

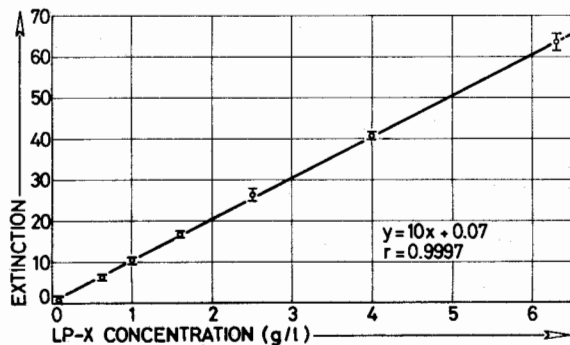


Fig. 4. Standard curve for LP-X, as determined by densitometry of the agar-electropherogram (Rapidophor) of LP-X preparations obtained from six different patients

The LP-X concentration in various samples can now easily be determined; results for typical samples are shown in Figure 5.

To establish the incubation time with polyanionic compounds, we measured LP-X positive sera with various concentrations of the abnormal lipoprotein. As indicated in Figure 6, precipitation was maximal after 30 min of incubation in all instances. Therefore all other measurements were done after 30 min of incubation.

The time required for LP-X quantitation by this technique is less than 1.5 h; one technician can easily quantitate as many as 40 samples within 2 h.

### Discussion

The LP-X test was earlier demonstrated to be a valid diagnostic tool to demonstrate or exclude cholestasis (9-15). In experimental cholestasis, LP-X may be detected and quantitated as early as 24 h after ligation of the common bile duct (18). For clinical needs, simple demonstration of LP-X seems to provide the most important information. However, certain questions, in particular those regarding the

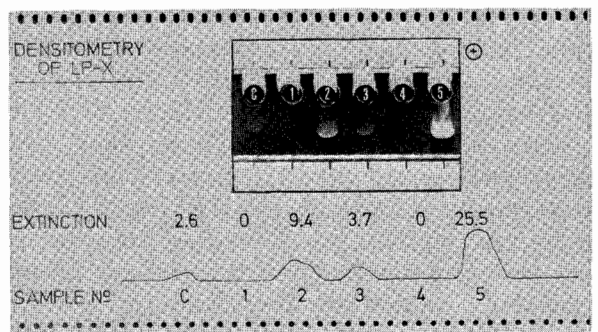


Fig. 5. Quantitation of LP-X in random samples

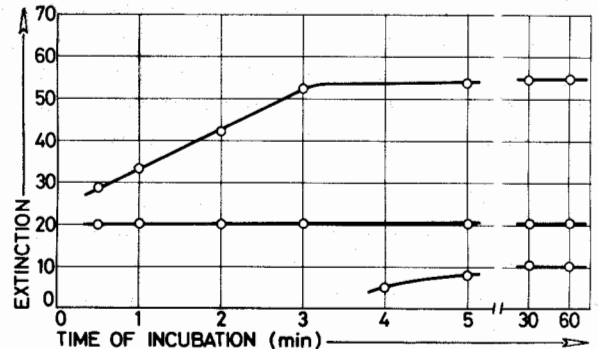


Fig. 6. Time curve for LP-X quantitation  
Incubation time for polyanionic compounds after agar electrophoresis

still unknown metabolism of this abnormal lipoprotein compound, may require exact quantitation.

The techniques for quantitation of LP-X that were described earlier (8, 20-22) had several disadvantages for routine use. For the semiquantitation by continuous dilution of the patient's serum (8) a specific antiserum was needed and a 2-h incubation was required after electrophoresis in agar.

Modifications of the Laurell and Mancini techniques for quantitation of LP-X (21) also required specific antisera, which are not readily available. In addition to possible problems in preparing the antibody-containing plates needed, continuous standardization of the system is required by running control sera of known LP-X concentration; these are difficult to obtain and to preserve over a longer period of time. These techniques also did not allow an exact quantitation with concentrations of less than 200 mg/liter for the Laurell technique and 300 mg/liter for the Mancini technique. Time required for these procedures ranged from 3 to 72 h. The quantitation of LP-X based on the measurement of phospholipids as a constant portion of LP-X in the cathodal area of the agar plates after electrophoresis (20) requires much technical work and permits exact quantitations only in concentrations above 500 mg/liter. The coefficient of variation for this technique was not given in the original paper, but in a comparable study (22) it was near 30%. The quantitative determination of LP-X described by Ritland (22) is based on an in

vitro equilibration of added radiolabeled cholesterol with all lipoproteins in the patient serum. From the percentage of radioactivity in LP-X obtained after agar electrophoresis, the concentration in serum was calculated. Although this technique provided a coefficient of variation of 10%, it requires too many technical steps and is certainly too time-consuming and laborious for routine work. Moreover, the upper and lower concentration limits for this method have not yet been well established. The advantage for routine and experimental work of the method described in the present paper is its simplicity and accuracy (CV, <5%) and the wide range of concentration in which LP-X can be assayed (60 mg to 6.3 g/liter). The method is easy and fast (up to 40 samples within 2 h by one person) and the standardization is done with the aid of a commercially available filter. All the equipment required for this technique is available and no additional preparations are needed. Lipoprotein-X in patients' sera may now be measured routinely.

Results of such studies may provide data on LP-X metabolism and possibly may contribute new information to clinical medicine that will enable the course of cholestatic liver disease to be followed.

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