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Note

Single-stage evaluation of serum lipoproteins by gel permeation using a specific fluorescent lipid probe

HILLA KNOBLER and MENAHEM FAINARU

Lipid Research Laboratory, Department of Medicine A, Kaplan Hospital, Rehovot 76-100 (Israel)

and

DAVID SKLAN*

Faculty of Agriculture, Hebrew University, P.O. Box 12, Rehovot 76-100 (Israel)

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The identification and treatment of individuals at risk from cardiovascular disease require evaluation of the circulating lipoprotein status. This has been traditionally carried out by ultracentrifugation of the plasma. This technique, however, is unwieldy, requires at least 18 h, and is limited in the number of samples that it can reasonably handle.

Recently, high-performance chromatography techniques to separate serum lipoproteins from plasma have been reported [1–3]. In this technique, monitoring absorbance at 280 nm is not sufficient for lipoprotein evaluation, as the amounts of the lipoproteins of lower molecular mass (M_r) are low compared with those of immunoglobulins and albumin, which elute with similar retention times. Some methods have involved separation of the eluate and performance of additional reactions, such as on-line determination of cholesterol and other lipids [2]. The equipment required, however, is costly and complex.

Nile red, a red phenoxazone dye, has fluorescence properties that allow it to behave as a hydrophobic probe, as the fluorescence intensity increases many-fold in the presence of hydrophobic compounds. Furthermore, Nile red stains with a degree of specificity in that the excitation and emission spectra of neutral lipids stained with Nile red show a blue shift compared with phospholipids. The intensity of Nile red fluorescence is similar for triglycerides and phospholipids, whereas the albumin-free fatty acid complex exhibits fluorescence some ten-fold less intense [4].

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This study reports on the use of Nile red for single-stage plasma lipoprotein evaluation by gel permeation chromatography.

EXPERIMENTAL

Serum samples were obtained from normal subjects and hyperlipidemic patients following overnight fast (12 h), and stored at 4°C until analysis within 1 week of sampling.

Very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were isolated from fasting normo-lipidemic human plasma by sequential ultracentrifigation [5] using a Beckman (Palo Alto, CA, U.S.A.) 50.3 Ti rotor in an L8-70 ultracentrifuge at 4°C at the following potassium bromide densities (g/l); 1.006 (18 h), 1.019–1.05 (18 h) and 1.063–1.21 (44 h). The lipoproteins thus obtained were washed, recentrifuged at their limiting density, and dialysed at 4°C against 0.15 *M* sodium chloride containing 0.1 m*M* EDTA for 24 h.

Nile red was prepared from Nile blue (Sigma, St. Louis, MO, U.S.A.) as described by Greenspan and Fowler [4], and 0.04 ml of a 50 μg/ml Nile red solution in acetone [4] were added per ml of aqueous solution or plasma. Fluorescence was determined in a fluorimeter (SLM-Aminico, Urbana, IL, U.S.A.).

Chromatography of plasma lipoproteins was carried out using a Superose 6 HR 10/30 (Pharmacia, Uppsala, Sweden) column eluted with 0.15 *M* sodium chloride–0.05 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.3 ml/min. The absorbance of the eluate was monitored at 280 nm, and fractions were collected.

Lipid classes were separated by thin-layer chromatography on silica gel after addition of triheptadecanoylglycerol and diheptadecanoyl phosphatidylcholine as internal standards [6], and fatty acid methyl esters were quantified by gas chromatography. Cholesterol was determined essentially as described by Searcy and Berquist [7].

RESULTS

Several characterized separated lipoproteins were stained with Nile red and separated on Superose 6 HR. Their absorbance was monitored at 280 nm, and the fluorescence pattern in the eluate was determined. The values of log M_r of the lipoproteins together with protein standards are shown in Fig. 1. The lipoproteins all eluted as single symmetrical peaks that were well separated from each other. Treatment with Nile red either before or after chromatography of the individual characterized lipoproteins did not effect the elution volume. Thus the Nile red fluorescence can be monitored for lipoprotein detection either in elution fractions or on-line with an appropriate detector.

The fluorescence elution profiles of normal sera stained with Nile red were examined and a typical example is shown in Fig. 2. together with the cholesterol and triglyceride elution patterns. The fluorescence peaks parallel the elution of triglyceride and cholesterol in the VLDL, LDL and HDL.

The use of the fluorescence elution profiles of serum samples from two hyper-

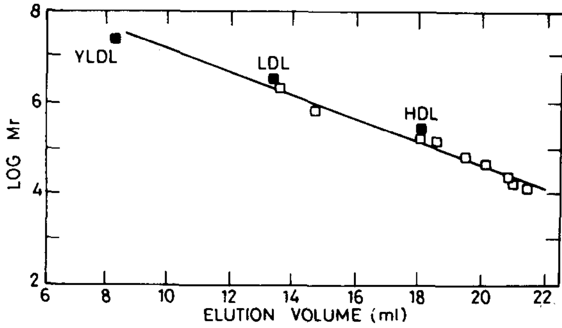

Fig. 1. Elution volumes of standard proteins and ultracentrifugally separated lipoproteins on the Superose 6 HR column. Thyroglobulin, immunoglobulin G, phosphorylase B, bovine serum albumin, ovalbumin, chymotrypsin, myoglobin and ribonuclease served as protein standards.

lipidemic patients is shown in Fig. 3. R.E., a 44-year-old male who suffers from ischemic heart disease, had serum levels of triglyceride of 710 mg% and cholesterol of 270 mg%. The elution profile of R.E. exhibits a much higher level of VLDL than is seen in normal subjects, with an additional incompletely separated shoulder between VLDL and LDL. The second patient was A.H., a 30-year-old male with a history of bouts of abdominal pain, compatible with recurrent pancreatitis. Serum from A.H. exhibited a thick creamy layer, and had a triglyceride level of 1280 mg% and a cholesterol level of 380 mg%. The chromatogram showed a pronounced fluorescent peak that eluted prior to VLDL, and a non-typical pat-

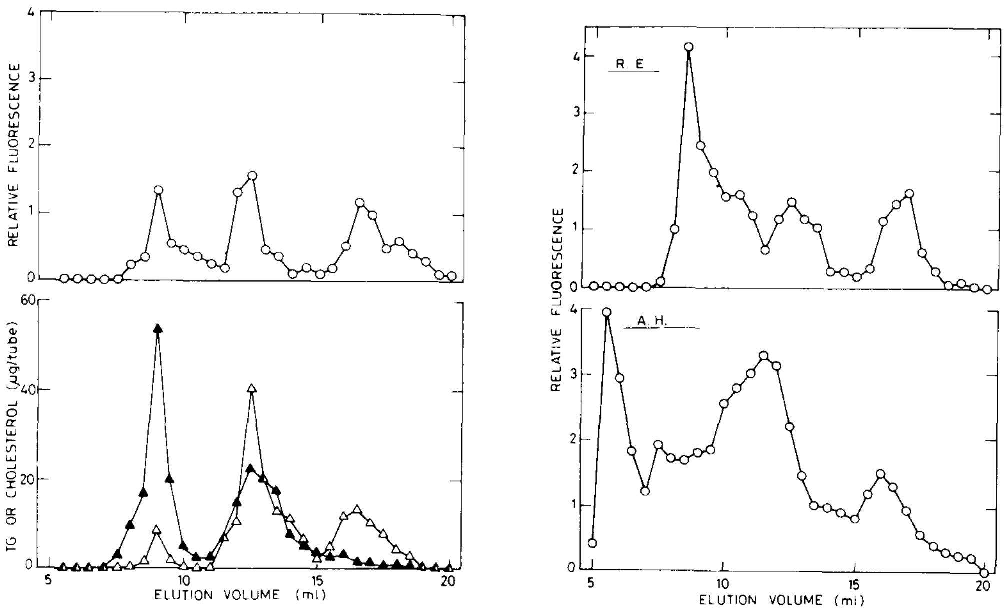

Fig. 2. Fluorescence (upper panel), triglyceride (lower panel, filled triangles) and cholesterol (lower panel, open triangles) profiles of normal serum separated on the Superose 6 HR column.

Fig. 3. Fluorescence profiles of two hyperlipidemic patients on Superose 6 HR.

tern of elution of VLDL and LDL with additional incompletely separated peaks between VLDL and LDL and HDL. Triglyceride and cholesterol elution (not shown) paralleled that of the fluorescence peaks.

DISCUSSION

Nile red staining allows qualitative visualization of lipoproteins in plasma by gel permeation of serum in a single-stage "on-line", using an appropriate fluorescence detector. Chromatography of separated lipoproteins stained with Nile red on Superose 6 HR resulted in fluorescence profiles identical with the absorption profiles. Use of this technique with whole plasma yielded fluorescence peaks that paralleled the triglyceride and cholesterol elution, but were not subject to interference from other non-lipoproteins. Albumin-free fatty acid was separated from HDL by the column, and no fluorescent peak of magnitude similar to that of the lipoproteins was observed in plasma at this retention volume. The Nile red staining techique can thus be used for qualitative evaluation of lipoproteins in serum in a single-stage procedure.

The clinical application of Nile red staining for lipoprotein evaluation is demonstrated in two hyperlipidemic patients. The lipoprotein patterns of two patients, R.E. and A.H., clearly differ from the normal. The serum of R.E., with a high level of triglyceride, exhibits major abnormalities compared with normal serum: a strikingly higher VLDL peak, as well as other, lesser differences. This pattern is suggestive of a Type IV hyperlipidemia. Another example is the chromatography of serum from A.H., who had an extremely high level of triglyceride. This revealed a considerably fluorescent peak eluting before VLDL, which was not apparent in normal serum. This suggests that in contrast to R.E., the high level of triglyceride in A.H. originated in part from particles larger than VLDL, which are probably chylomicrons. This is compatible with the creamy layer found in this overnight refrigerated serum. These findings, together with the clinical history, are suggestive of Type V hyperlipidemia.

While the elution profile obtained from normal serum disclosed symmetrical peaks, the serum of both patients revealed peaks that were not discrete but overlapped. These additional lipoproteins may represent transfer fractions between lipoprotein classes, as has been previously reported by Eisenberg et al. [8] in subjects with severe hypertriglyceridemia. This indicates an additional use of the Nile red staining, namely to identify sub-populations of lipoproteins not readily visualized using conventional lipoprotein separation methods.

These examples illustrate the use of staining with Nile red to identify the distribution of lipoprotein classes in normal serum, in disease states and during therapy. The separation took ca. 1 h, and results are immediately apparent provided an appropriate fluorescence detector is used.

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