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Note

New principle for the separation of plasma lipoprotein lipids without ultracentrifugation

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The diagnosis and therapeutic management of disorders in lipid metabolism requires detailed yet simple analytical procedures for the determination of plasma lipid and lipoprotein (LP) lipid concentrations [1—6].

Lipid extraction from serum samples followed by chemical lipid determination is time-consuming and relatively large sample volumes are needed. In a recent report [7], however, the direct application of 0.5-μl plasma samples to high-performance thin-layer plates prior to lipid separation by thin-layer chromatography (TLC) was shown to be feasible. The serum proteins were denatured by methanol.

The separation of plasma LP fractions by ultracentrifugation followed by lipid analysis using chemical methods is also widely used. Although accepted as the reference method for LP fractionation, this procedure is restricted to clinics and institutes specialized in this technique. In addition, large sample volumes are required. Therefore, a procedure has been developed for the quantitative enzymatic determination of cholesterol in LP fractions separated by electrophoresis on agarose gel [8—10]. The agarose samples containing the LP fractions of 15 μl capillary plasma were dissolved in HCl and, after neutralization, a commercial test combination was used for the enzymatic determination of cholesterol. No influence of the HCl on cholesterol was observed.

These principles (applying serum or plasma directly to commercial TLC plates, and dissolving agarose samples containing LP lipids in HCl) have now been applied to a combined electrophoresis and TLC procedure for the separation of plasma lipids and LP lipids on a microscale and without ultracentrifugation.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. TLC plates were pre-coated with silica gel 60 without fluorescent indicator (20 × 20 cm; Merck, Darmstadt, G.F.R.). [1α, 2α-^{3}H]Cholesterol (>30,000 mCi/mmole) and Insta-Gel liquid scintillation agent were supplied by Amersham/Buchler, Braunschweig, G.F.R.

Electrophoresis

The LP fractions of 20 μl of freshly prepared blood serum or plasma were separated on agarose gel and made visible by opalescence as previously described [8].

Direct application of the agarose samples containing the LP fractions to TLC plates

The opalescent LP fractions were cut with a scalpel. After evaporation of adherent moisture, the samples were transferred to the TLC plate. Moisture in the agarose gel was evaporated in vacuo for a few minutes. Caution was required to ensure that the agarose samples did not lift from the silica gel layer. This was guaranteed as long as a white circle was visible around the agarose sample on the silica gel layer. When the white circle was clearly visible, the vacuum was released very carefully. The agarose samples appeared as a transparent film. Immediately, a few microlitres of concentrated HCl were deposited on each sample, thereby destroying the agarose structure. The HCl was evaporated under a stream of cold air from a hair dryer.

Direct application of the serum or plasma samples to the TLC plates

A few microlitres of concentrated HCl were deposited on the TLC plate prior to and after the application of 20 μl of sample. The HCl was evaporated under a stream of cold air.

Separation and detection of lipids

Lipids were separated and detected as described previously [7].

Radiochemical examination of the quality of the HCl procedure

[^{3}H]Cholesterol (20 μCi) was dissolved in benzene and applied to a piece of filter paper [11]. After evaporation of the solvent, the piece of paper was added to 3 ml of freshly prepared serum or plasma. Incubation was carried out for 1 h at 37°. The samples labelled with [^{3}H]cholesterol were analysed by electrophoresis and TLC as described above. The lipid spots were scraped from the TLC plates and transferred to liquid scintillation counting vials containing 10 ml Insta-Gel. For measuring radioactivity of the total serum, 20-μl samples were mixed with 10 ml Insta-Gel. Radioactivity was counted using a liquid scintillation spectrophotometer (Packard, Downers Grove, Ill., U.S.A.) [12].

RESULTS AND DISCUSSION

Principle of the procedure and its radiochemical examination

In a recent study, serum samples could be applied directly to TLC plates prior to the separation of the lipids. The serum proteins were denatured by methanol [7]. In the present study, the same end was attained by the addition of concentrated HCl to the serum sample after its direct application to the TLC plate. In Fig. 1, the lipid patterns of a serum sample and of the corresponding lipid extract are shown. No marked differences are apparent between the samples (Fig. 1A). From this lipid extract, free and esterified cholesterol and triacylglycerols were isolated by preparative TLC. Following exposure of these lipids to the HCl procedure on the TLC plate, re-chromatography did not show changes due to HCl (Fig. 1B).

Fig. 2 demonstrates the lipid patterns of a sample with known lipid concentrations (Precilip) and of a serum of a subject with its corresponding LP fractions. The separation of the neutral lipids was satisfactory. Semiquantitation of the lipids by visual examination compared to the known lipid content of Precilip is possible.

For radiochemical examination of the procedure, serum was incubated with [^{3}H]cholesterol for 1 h at 37°. Of the ^{3}H-label, 50—60% was taken up by the serum LPs and distributed between the LP fractions, as demonstrated in

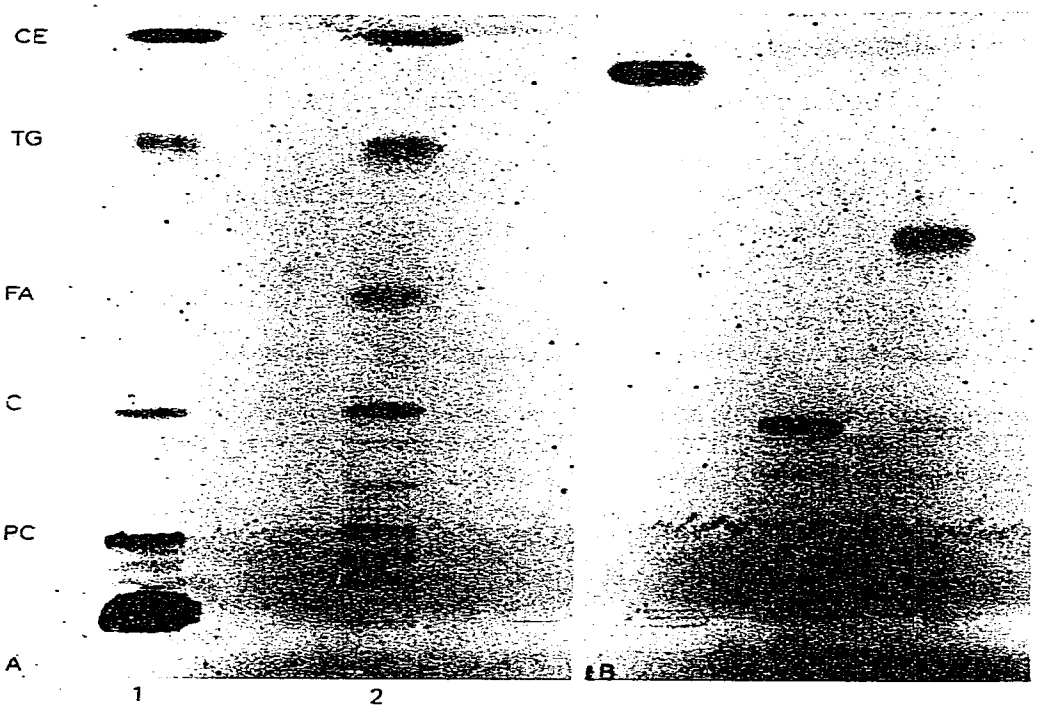

Fig. 1. Denaturation of serum lipids with HCl on the TLC plates. Twenty microlitres of serum (position 1) and the corresponding amount of lipid extract (position 2) were applied to the TLC plate and the HCl procedure was carried out (A). From this lipid extract, cholesterol esters (CE), free cholesterol (C) and triacylglycerols (TG) were prepared by preparative TLC and exposed to HCl on the plate (B). FA, fatty acids; PC, phosphatidyl choline.

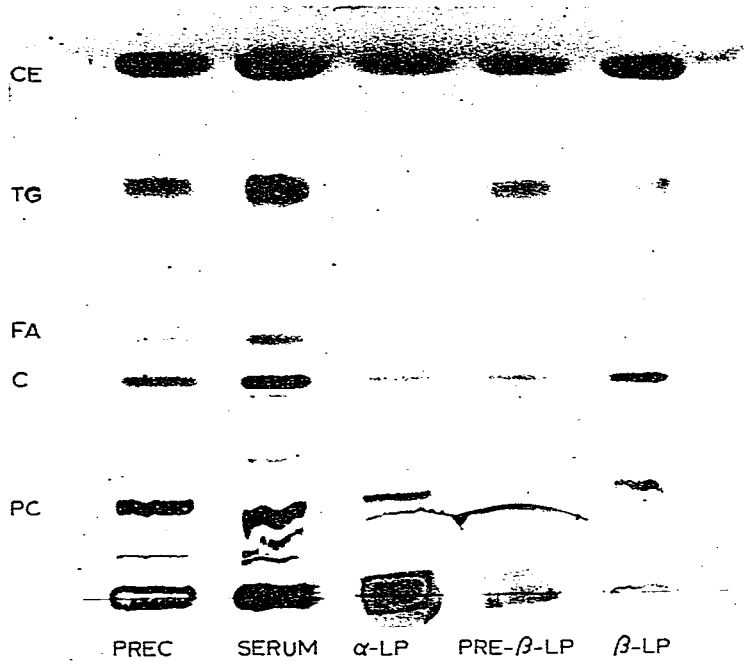

Fig. 2. Direct application of agarose samples containing LPs to the TLC plate. The LP fractions were obtained by electrophoresis on agarose gel and applied to the TLC plate followed by the HCl procedure. After TLC the distribution of the serum lipids among the LP fractions can be examined. Semiquantitative evaluation is possible by comparison with a sample (Precilip = PREC) containing known lipid concentrations (CE = 2.16 mmole/l, C = 0.86 mmole/l, and TG = 0.63 mmole/l). Abbreviations as in Fig. 1.

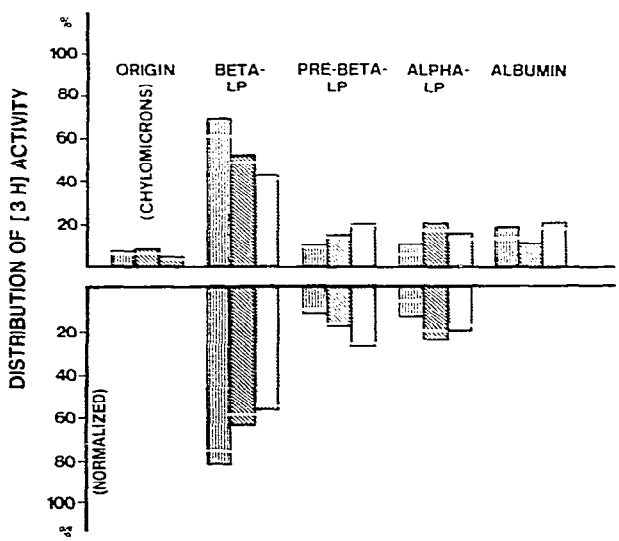

Fig. 3. Labelling of serum LPs. Serum was incubated with [^{3}H]cholesterol for 1 h at 37° prior to electrophoresis. The fractions were cut and radioactivity measured. Two samples from each of three subjects were studied. Normalized scale: distribution of radioactivity among LP fractions alone.

Fig. 3. Traces of label were found at the origin of the electropherogram. The remainder was preferentially taken up by the β-LPs and to a much lesser extent by the other fractions. ^{3}H-Label found in albumin was probably due to α-LPs included in this fraction.

TLC and counting of ^{3}H-activity in the different areas of the TLC layer showed that approximately 85—95% of the [^{3}H]cholesterol was recovered as free cholesterol with high precision (coefficient of variation, C.V. = 2—3%), and 10% as esterified cholesterol (C.V. <15%) (Table I). Only trace amounts of radioactivity were found in the other areas and at the origin of the chromatoplates. It was postulated from these results that negligible amounts of free and esterified cholesterol remain at the origin during TLC. Elution of the origin spots followed by re-chromatography showed that practically no cholesterol or other neutral lipids had remained there during the TLC procedure.

TABLE I

RADIOCHEMICAL EXAMINATION OF THE COMBINED ELECTROPHORESIS AND TLC PROCEDURE

The serum LPs labelled with [^{3}H]cholesterol were separated by electrophoresis. The LP fractions were applied directly to the TLC plates and, after HCl treatment of the samples, TLC was carried out. The cholesterol esters (CE), free cholesterol (C) and the areas marked by brackets were scraped from the plate and radioactivity was counted. Data are expressed as a percentage of total radioactivity; coefficients of variation (C.V., %) are given in parentheses. Values are averages of 10 samples ± S.D.

	Serum	β-LP	pre-β-LP	α-LP
CE	6.9 ± 0.7 (10.1)	2.4 ±0.2 (8.3)	3.2 ± 0.5 (15.6)	12.2 ± 1.6 (13.1)
	1.2/0.9	1.2/0.9	1.7/2.5	1.4/1.0
C	91.6 ± 1.6 (1.7)	95.9 ± 1.4 (1.5)	92.9 ± 2.1 (2.3)	85.6 ± 2.6 (3.0)
	1.3 ± 0.1 (7.7)	1.5 ± 0.2 (13)	1.4 ± 0.2 (14)	1.3 ± 0.2 (11)

Application to the evaluation of esterifying activity in LP fractions of ten healthy subjects

The procedure described above was extended to the analysis of the serum of 10 healthy (control) subjects. Fig. 4 demonstrates that the serum LP fractions exchanged their cholesterol with [^{3}H]cholesterol during the incubation. Up to 10% of this [^{3}H]cholesterol was esterified and mainly localized in the α-LPs (Fig. 4A). The distribution of free [^{3}H]cholesterol among the LP fractions represents to a certain extent the total cholesterol moieties in these fractions (Fig. 4B). The [^{3}H]cholesterol esters, however, were present in equal amounts in the α- and β-LPs independent of the very different amounts of cholesterol in these fractions. This result is consistent with the observation that plasma esterifying activity due to lecithin:cholesterol acyl transferase (LCAT) is mainly localized in the α-LPs [13—18], and that the β- and pre-β-LPs participate in this process, perhaps by exchanging the newly synthesized cholesterol esters with the α-LPs [19].

Typical LP patterns in control and hyperlipidemic subjects

The procedure was applied to the separation of the plasma lipids and LP lipids of healthy and hyperlipidemic subjects. Fig. 5 presents the lipid patterns

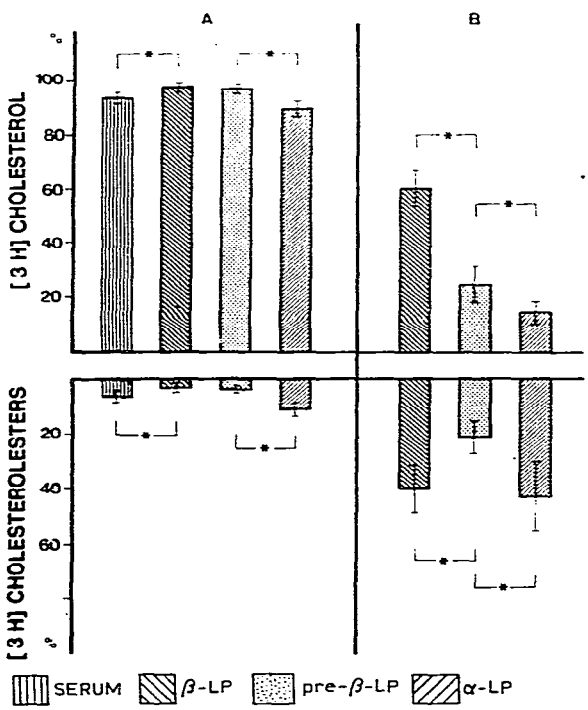

Fig. 4. Cholesterol esterifying activity in ten healthy subjects. Serum of ten healthy subjects was incubated with [^{3}H]cholesterol for 1 h at 37°, after which the combined electrophoresis and TLC procedure was carried out. Radioactivity was counted in the free and esterified cholesterol. A: Proportion of free and esterified [^{3}H]cholesterol in each sample. B: Distribution of free [^{3}H]cholesterol and [^{3}H]cholesterol esters among the three LP fractions. Asterisks (*) indicate significant differences.

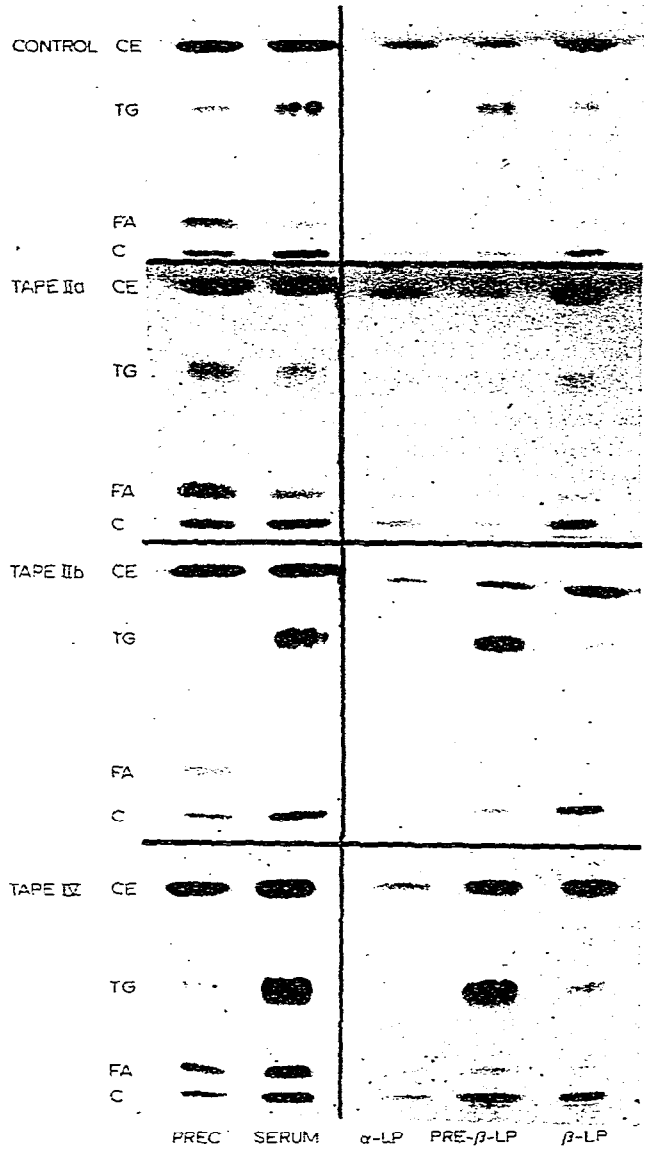

Fig. 5. Typical lipid patterns. The neutral lipids of a known sample (Precilip ≈ PREC) are compared with those in serum and the corresponding LPs of four subjects. Results on one healthy (control) subject and on patients with three types of hyperlipidemia are shown. The lower parts of the chromatograms have been deleted. Abbreviations as in Fig. 1.

of a healthy (control) subject, and of Type IIa, Type IIb and Type IV hyperlipidemic patients. The contents of the plasma lipids and their distribution among the LP fractions are compared to a sample with known amounts of lipid. Visual examination of the chromatoplates gives information on the qualitative and semiquantitative lipid composition of the LP fractions (the quantitation of these lipids is in progress). The distribution of the serum cholesterol among the LP fractions is consistent with data previously published [8]. This distribution is quite similar for normal and Type II subjects, regard-

less of the total cholesterol level. The pre-β-LPs of Type IV hyperlipedemic patients, however, carry larger amounts of cholesterol. In all types of serum, the triacylglycerols are carried predominantly by the pre-β-LPs.

CONCLUSIONS

Serum LP fractions obtained by electrophoresis on agarose gel were applied directly to the TLC plate and HCl used to dissolve the agarose structure prior to TLC of the lipids. The quality of the procedure was examined by radiochemical analysis of [^{3}H] cholesterol-labeled LP fractions.

The method was applied to the examination of the esterifying activity of LP fractions from healthy subjects. Furthermore, typical lipid patterns of serum and serum LP fractions were demonstrated. The visual examination of the chromatoplates gave satisfactory results. The quantitation of the lipids is in preparation.

This new micro-procedure can serve for further studies in phenotyping hyperlipidemias, in therapeutic management of the different dyslipoproteinemias, and in identifying subjects with a high risk of atherogenesis.

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