notes on methodology

Localization of lipoprotein unesterified cholesterol in nondenaturing gradient gels with filipin

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Summary A method is described for the staining of lipoprotein unesterified cholesterol in nondenaturing polyacrylamide gradient gels with the fluorescent polyene antibiotic, filipin. The sensitivity of the filipin stain was comparable to that of oil red O and Coomassie R250 in terms of the amount of lipoprotein applied. Filipin successfully stained discoidal complexes of apoA-I–phosphatidylcholine–cholesterol, which in turn were stained poorly with oil red O. The potential for the identification of unesterified cholesterol-enriched lipoprotein subclasses was demonstrated. — **Lefevre, M.** Localization of lipoprotein unesterified cholesterol in nondenaturing gradient gels with filipin. *J. Lipid Res.* 1988. **29:** 815–818.

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Nondenaturing gradient gel electrophoresis (GGE) has been demonstrated to be a convenient and sensitive method for the identification of multiple HDL and LDL subclasses in human plasma (1–3). Typically, ultracentrifugationally isolated samples are electrophoresed overnight and the distribution of the separated lipoproteins is visualized by protein staining with Coomassie R250. On occasion, it is desirable to determine the distribution of the lipoproteins without prior ultracentrifugation. In this instance one must resort to the use of a lipid stain, usually oil red O, to visualize the lipoproteins. However the use oil red O is not without problems. It is relatively difficult to make and store and, when used as a stain for GGE, it forms a precipitate on the surface of the gels. Additionally, oil red O is relatively specific for neutral lipids (cholesteryl esters and triglycerides), staining the more polar lipids (unesterified cholesterol and phospholipids) poorly. This latter point can be a disadvantage when one is interested in examining nascent or abnormal lipoproteins that may be substantially enriched in polar lipids (i.e., discoidal particles).

The polyene antibiotic, filipin, specifically binds to 3β-hydroxysterols (4, 5). It has been used in a number of studies for the histochemical identification of unesterified cholesterol in atherosclerotic lesions (6). In this report, we describe the use of filipin for the staining of unesterified cholesterol in lipoproteins separated by GGE.

MATERIALS AND METHODS

Blood from volunteers who had fasted overnight was collected into tubes containing EDTA. Plasma was collected by centrifugation at 1500 *g* for 20 min. DTNB was added at a final concentration of 1.4 mM to inhibit LCAT. Dog prenodal peripheral lymph was collected from the hindlimb of fasted foxhounds as previously described (7). Discoidal complexes containing human apoA-I, egg PC, and unesterified cholesterol were prepared by the cholate dialysis method as described by Jonas, Sweeny, and Herbert (8). In preparing the complexes, final weight ratios of 2.79:0.138:1.0:1.54 for egg PC–cholesterol–apoA-I–sodium cholate were used.

Human lipoproteins were isolated by ultracentrifugation following adjustment of plasma to d 1.21 g/ml by addition of solid KBr. Samples were centrifuged for 48 hr in a Beckman SW-60 rotor at 100,000 *g* operated at 10°C. Lipoproteins were dialyzed against several changes of PBS.

In preparation for gradient gel electrophoresis, samples were mixed with 0.25 volumes of electrophoresis buffer (90 mM Tris base, 80 mM boric acid, 3 mM EDTA, pH 8.35) containing 40% sucrose and 0.01% bromphenyl blue. Following pre-electrophoresis for 15 min at 125 V, the samples were applied to standard Pharmacia 12-place well-formers and electrophoresed as described by Blanche et al. (1) on Pharmacia PAA 4/30 gradient gels at 125 V for 24 hr at 10°C. Upon completion of the electrophoresis, the discoidal particles and isolated lipoproteins were stained overnight for protein with 0.05% Coomassie R250 in methanol–acetic acid–water 45:10:45. The gels were destained with several changes of methanol–acetic acid–water 25:10:65. Neutral lipids were stained overnight at 40°C with 0.04% oil red O in 60% ethanol. Gels were destained with several changes of methanol–acetic acid–water 25:10:65.

Staining of unesterified cholesterol with filipin was conducted as follows. Following electrophoresis, gels were fixed for 1 hr with 10% TCA. The gels were then incubated with several changes of PBS to remove the TCA. Filipin stain was prepared by dissolving 10 mg of filipin (Sigma) in 1 ml of dimethylformamide. This was then added to 100 ml of PBS containing 0.1% NaN_3. Gels were stained overnight with the filipin stain and destained with several changes

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; GGE, gradient gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DTNB, 5, 5'-dithiobis(2-nitrobenzoic acid); LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; apo, apolipoprotein; TCA, trichloroacetic acid; PBS, phosphate-buffered saline.

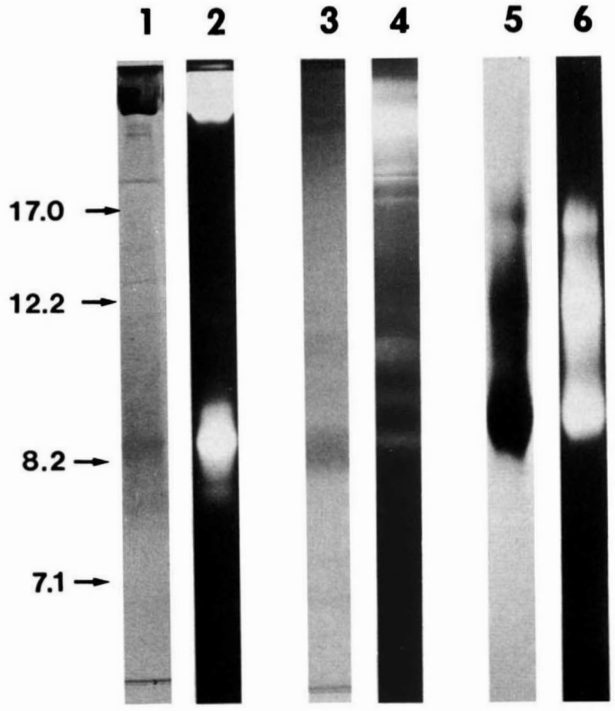

Fig. 1. Comparison of filipin and oil red O- or Coomassie R250-stained nondenaturing gradient gels (PAA 4/30). Lane 1, human plasma (20 μl) stained with oil red O. Lane 2, human plasma (15 μl) stained with filipin. Lane 3, dog prenodal peripheral lymph (80 μl) stained with oil red O. Lane 4, dog prenodal peripheral lymph stained with filipin. Lane 5, apoA-I-egg-PC-cholesterol discoidal complexes (15 μg of protein) stained with Coomassie R250. Lane 6, apoA-I-egg-PC-cholesterol discoidal complexes stained with filipin. Approximate migration and Stokes radii of molecular weight standards are indicated on the left. Slight variations in lipoprotein migration and gel length between paired samples is due to differential swelling of the gel in the presence (oil red O and Coomassie R250) or absence of methanol (filipin) in the destain solution.

of PBS containing 0.01% NaN_3. Care was taken to monitor the extent of destaining since extended destaining could result in total loss of filipin from the gel. The filipin stain was saved, stored at 4°C, and could be reused several times within a week.

Gels were scanned with a Corning model 760 Fluorometer/Densitometer. Individual scans were digitized with a Hewlett Packard 7475A graphics plotter interfaced to a microcomputer. The digitized scans were analyzed by specialized software that reduced the data to provide a report of percent distribution of stain as a function of HDL subfraction as defined for gradient gel electrophoresis by Nichols, Blanche, and Gong (2). Values for $(HDL_{2a})_{gge}$ and $(HDL_{2b})_{gge}$ were added to provide estimates of HDL_2 as a percent of total HDL. HDL_2 total and unesterified cholesterol were independently estimated by the precipitation method of Gidez et al. (9). Total and unesterified cholesterol were assayed enzymatically (10).

RESULTS AND DISCUSSION

Filipin staining of nondenaturing gradient gels was found to be a convenient method for localizing lipoproteins. As opposed to oil red O, the preparation of the stain was simple and the staining and destaining of the gels could be accomplished without much difficulty. The filipin stain was about as sensitive as oil red O or Coomassie R250 in terms of amount of lipoprotein applied to the gel **(Fig. 1).**

The ability of filipin to specifically stain unesterified cholesterol may allow one to tentatively identify subpopulations of lipoproteins that may be relatively enriched in unesterified cholesterol. It is, therefore, noteworthy that the scans of filipin-stained gels gave profiles different from their oil red O- or Coomassie R250-stained counterparts **(Fig. 2).** In the sample of human plasma (Fig. 1, lanes 1 and 2; Fig. 2A), a specific subpopulation of HDL with a modal diameter of 8.4 nm appears to be preferentially stained by filipin. Similarly, in dog prenodal peripheral lymph (Fig.

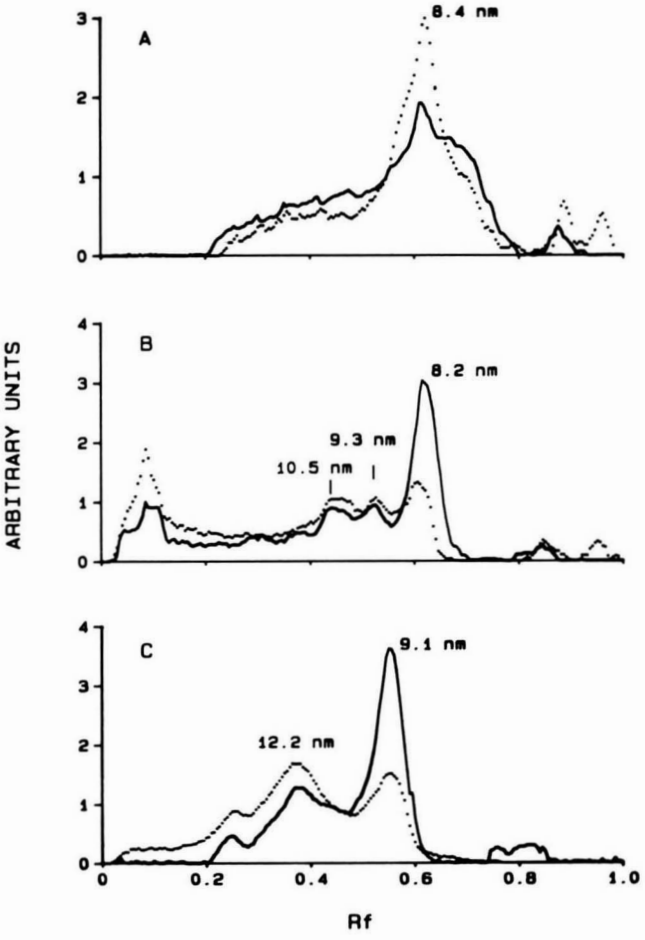

Fig. 2. Scans of the gels shown in Fig. 1. Human plasma (A); dog prenodal peripheral lymph (B); and apoA-I-egg-PC-cholesterol discoidal complexes (C). In panel A, only the HDL region is shown; (—), oil red O (panels A and B) or Coomassie R250 (panel C); (···), filipin.

1, lanes 3 and 4; Fig 2B), particles with modal diameters of 9.3 and 10.5 nm also appear to stain 10–20% better with filipin than with oil red O. This is of particular interest since it is known that dog prenodal peripheral lymph contains both spherical HDL enriched in free cholesterol as well as nascent discoidal particles (11).

The ability to detect discoidal structures with filipin was demonstrated with discoidal complexes of apoA-I, egg PC, and cholesterol prepared by the cholate dialysis method. While these complexes were well stained with both Coomassie R250 and filipin (Fig. 1, lanes 5 and 6), they were barely detectable by oil red O (gel not shown). Furthermore, Coomassie R250 and filipin differentially stained the various subpopulations comprising these complexes. Thus, while the majority of Coomassie R250 staining material was localized to a population of particles with a modal diameter of 9.1 nm, the major filipin staining population had a modal diameter of 12.2 nm (Fig. 2C). The preferential filipin staining of the larger discoidal structure is consistent with a recent report by Nichols et al. (12) which indicated that the larger apoA-I–phospholipid–cholesterol discoidal complexes were significantly enriched in unesterified cholesterol.

Filipin staining of HDL subpopulations in whole plasma was further compared with oil red O staining of whole plasma, with filipin and Coomassie R250 staining of isolated lipoprotein fractions, and with the sequential precipitation method for estimation of HDL_2 cholesterol in an additional five human subjects. **Fig. 3** shows that estimates of HDL_2 (as a percentage of total HDL) by filipin staining of whole plasma correlate well with percent HDL_2 total cholesterol ($R = 0.993$) and unesterified cholesterol ($R = 0.911$) as determined by the precipitation method. However, while the correlations are good, the filipin staining method tended to underestimate the relative amount of HDL_2 total cholesterol and overestimate the relative amount of HDL_2 unesterified cholesterol. These differences are not unexpected since these two methods rely upon completely different physical properties for estimation of HDL_2. It should be pointed out that estimates for the relative amount of HDL_2 based on oil red O staining of gradient gels correlated less well with the relative amount of HDL_2 total cholesterol ($R = 0.718$) than did the filipin method. Additionally, filipin staining-derived estimates of the relative amount of HDL_2 correlated well ($R = 0.936$) with estimates derived from Coomassie R250-stained gradient gels of the corresponding isolated lipoproteins (Fig. 3C). Estimates of HDL_2 by the filipin method are reproducible; repeated analysis of samples yielded average intra- and inter-assay coefficients of variation of 4.1% and 12.4%, respectively.

Additional comparisons of the filipin staining method with other staining techniques is provided in **Table 1.** The percent distribution of staining material in either whole plasma or isolated lipoproteins is presented as a function of HDL subfractions as defined for gradient gel electrophoresis by Nichols et al. (2). Three observations can be made. First, as previously indicated, the HDL subfraction distribution as estimated by filipin staining of whole plasma agrees quite well with values obtained by Coomassie R250 staining of isolated lipoprotein fractions. Second, again as previously suggested, a specific subpopulation of HDL, $(HDL_{3a})_{gge}$, stains significantly better with filipin than with oil red O, which suggests a relative enrichment of this subpopulation with unesterified cholesterol. And finally, the filipin staining distribution for the isolated lipoprotein fractions is significantly different from that obtained in the original plasma. While previous studies have clearly shown a redistribution of apolipoproteins following ultracentrifugation (13, 14), these data suggest that alterations in surface lipids may also occur. The limited number of subjects examined in this preliminary study precludes us from drawing any definitive conclusions. However, it is clear that

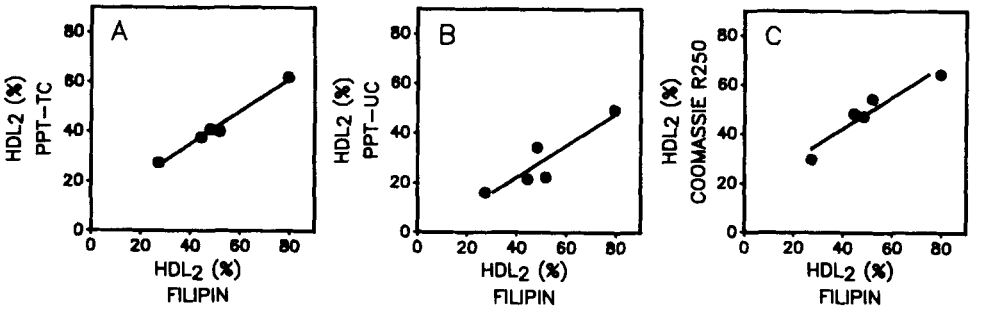

Fig. 3. Comparisons of filipin staining method with other methods for the estimation of HDL_2. Filipin staining of whole plasma from five individual donors after electrophoresis on nondenaturing gradient gels was accomplished as described in the text. The stained gels were scanned, digitized, and the data were converted to percent staining intensity in the region comprising HDL_2 ($(HDL_{2b})_{gge}$ and $(HDL_{2a})_{gge}$). The values obtained by the filipin method were correlated with values derived by the precipitation method of Gidez et al. (9) following assays for total cholesterol (PPT-TC; panel A) and unesterified cholesterol (PPT-UC; panel B). In addition, lipoproteins ($d < 1.21$ g/ml) were isolated from each individual, subjected to nondenaturing gradient gel electrophoresis, and stained for protein with Coomassie R250. Following scanning and subsequent data reduction, the values obtained by the Coomassie R250 method were correlated with values obtained by filipin method (conducted on the original plasma) (panel C).

TABLE 1. HDL subpopulation estimations by filipin, oil red O, and Coomassie R250 staining methods

Sub-population	Filipin (Plasma)	Oil Red O (Plasma)	Filipin (d<1.21 g/ml)	Coomassie R250 (d<1.21 g/ml)
			%	
HDL_{2b}	28.4 ± 20.3	35.0 ± 12.3	41.5 ± 19.9[a]	27.3 ± 12.5
HDL_{2a}	21.8 ± 3.4	23.4 ± 4.6	24.4 ± 7.7	21.5 ± 5.8
HDL_{3a}	28.7 ± 8.7	22.4 ± 7.1[a]	24.8 ± 8.9	30.4 ± 6.5
HDL_{3b}	14.6 ± 7.0	13.6 ± 6.5	7.3 ± 7.5[a]	15.7 ± 6.8
HDL_{3c}	6.5 ± 4.1	7.1 ± 1.7	2.5 ± 2.5[a]	5.1 ± 3.1

Plasma samples or isolated lipoproteins from five subjects were electrophoresed on nondenaturing gradient gels and then stained with either filipin, oil red O, or Coomassie R250 as described in the text. The gel scans were digitized and the data were reduced to provide a report of relative amount of staining material as a function of HDL subpopulation. HDL subpopulation designations were defined as described by Nichols et al. (2) for the gradient gel system.

[a] Significantly different ($p < 0.05$) from filipin-stained plasma gels as determined by analysis of variance followed by Fisher's least significant difference multiple range test. Data are mean ± SD.

the filipin staining method has the potential to provide information on lipoprotein distribution and composition which may then be confirmed using more traditional technologies.

In summary, a method has been described for the specific staining of free cholesterol in nondenaturing gradient gels using the polyene antibiotic, filipin. Its ease of use makes it a convenient method for the identification of lipoprotein subfraction by GGE without prior ultracentrifugation of the sample. Populations of lipoproteins, not easily detected by oil red O by virtue of a low neutral lipid content, may be more readily identified with the filipin stain. Finally, as suggested by the examples provided above, the use of filipin staining in conjunction with Coomassie and oil red O staining may be used to tentatively identify specific subpopulations of lipoproteins that are relatively enriched in unesterified cholesterol.

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