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DETECTION OF AN ABNORMAL LIPOPROTEIN IN A LARGE COLONY OF PEDIGREED BABOONS USING HIGH-PERFORMANCE GEL EXCLUSION CHROMATOGRAPHY

MARY CARR WILLIAMS

Southwest Foundation for Biomedical Research, San Antonio, TX 78284 (U.S.A.)

JIM LEE KELLEY

The University of Texas Health Science Center, San Antonio, TX 78284 (U.S.A.)

and

RAMPRATAP SINGH KUSHWAHA*

Southwest Foundation for Biomedical Research, P.O. Box 28147, San Antonio, TX 78284 (U.S.A.)

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SUMMARY

High-performance gel exclusion chromatography was used to detect an abnormal lipoprotein in a large colony of baboons. Serum obtained from fasting baboons was adjusted to density 1.21 g/ml and ultracentrifuged to obtain lipoproteins. A small fraction (equivalent to 50 μl serum) was separated using a gel filtration column (TSK 4000 SW) or a combination of TSK 4000 PW and TSK 3000 PW columns. The unusual lipoprotein was detected either as a distinct peak between low- and high-density lipoproteins or as a distinct shoulder to the high-density lipoprotein peak. In some baboons the unusual lipoprotein was present on both chow and atherogenic diet, but in most cases it was induced by feeding an atherogenic diet.

INTRODUCTION

The composition and levels of plasma lipoproteins in humans and animals are affected by a number of genetic and environmental factors. Recently a special class of lipoproteins has been detected in some baboons upon feeding a diet rich in cholesterol and saturated fats. These abnormal lipoproteins accumulate in the density region between low-density (LDL) and high-density (HDL) lipo-

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proteins corresponding to a flotation rate of $F^{\circ}_{1.20}$ 9—28 and, therefore, have been designated as F° 9—28 lipoproteins by Nichols et al. [1]. The lipid and apolipoprotein compositions of these abnormal lipoproteins are not well characterized; however, they seem to be rich in apolipoprotein A-I and E. The F° 9—28 phenotype appears to be genetically transmitted.

Plasma lipoproteins differ in their hydrated densities [2], electrophoretic mobilities [3,4], and particle size [5]. Based on these characteristics a number of methods have been used for the separation and characterization of the plasma lipoprotein profile of an individual. Among these methods are the preparative ultracentrifuge technique using sequential density centrifugation [6], the vertical rotor [7], or density gradient ultracentrifugation [8,9], low-pressure gel permeation chromatography using agarose as the separation medium [10,11], and electrophoretic techniques [12—14]. Although the preparative ultracentrifuge, column chromatographic, and electrophoretic techniques are very reliable for a limited number of samples, they cannot be used to screen a large number of animals. Since high-performance liquid chromatography (HPLC) using gel permeation columns of the TSK-Gel type has been shown to be effective for rapid profiling of human [15—20] and monkey [21] plasma lipoproteins, we applied and further developed this technique for this study.

MATERIALS AND METHODS

Animals and diets

A group of pedigreed baboons consisting of 400—500 animals was used for these studies. These animals varied from 1 to 10 years of age. Animals were first screened while they were on the chow diet (Ralston Purina, St. Louis, MO, U.S.A.). They were again screened on an atherogenic diet, rich in cholesterol and saturated fat, at the seventh and eighth week. The composition of these diets is given in Table I.

TABLE I

COMPOSITION OF CHOW AND ATHEROGENIC DIETS

Nutrients	Chow diet*	Atherogenic diet**
Carbohydrates (% kcal)	62	39
Protein (% kcal)	28	21
Fat (% kcal)	10	40
Energy (kcal per 100 g diet)	329	377
Cholesterol (mg/kcal)	0.03	1.7

*Baboon chow manufactured by Ralston Purina.

**Atherogenic diet was prepared by mixing 79.1% (dry weight basis) of Purina monkey meal 25-5045-6 (a special mix with no added fat, dehydrated alfalfa, sodium chloride, ascorbic acid or retinyl acetate) with dried egg yolk (4.8%), lard (14.3%), sodium chloride (1.1%), retinyl acetate (0.001%), ascorbic acetate (0.1%), and cholesterol (0.5%).

Isolation and characterization of standard lipoproteins

For isolation of standard lipoproteins, blood was drawn from fasting animals in tubes containing EDTA (1 mg/ml). The plasma was separated using low-speed centrifugation. Lipoproteins were isolated by sequential ultracentrifugation (Beckman L5-50) as described by Kushwaha and Hazzard [22] using densities for very-low-density lipoproteins (VLDL), LDL, and HDL as 1.006, 1.019—1.063, 1.063—1.21 g/ml, respectively, using a 50.3 Ti rotor. The densities were adjusted using solid potassium bromide. The lipoproteins (upper 2.5-ml layer) were obtained by slicing the tube. To detect if the lipoprotein fractions isolated by the sequential ultracentrifugation were pure fractions, their electrophoretic mobility and apoproteins were characterized. The lipoproteins had single bands corresponding to their characteristic electrophoretic mobility [4] on agarose gel electrophoresis. The apoproteins were characterized by separating delipidated lipoproteins [23] using 3.5% and 10% polyacrylamide gel electrophoresis (PAGE) containing 0.1% sodium dodecyl sulfate (SDS) [24]. The VLDL and LDL fractions had apo-B as their major protein, while HDL had apo-A-I and apo-A-II as its major proteins. Albumin was present in HDL in minor amounts even after washing once.

Preparation of lipoproteins for HPLC separation

Fasted animals (16—20 h) were bled to obtain 3 ml of blood in a tube containing EDTA (1 mg/ml). The blood was centrifuged in a low-speed refrigerated centrifuge to separate plasma. A 0.5-ml aliquot of plasma was adjusted to a density of 1.21 g/ml and ultracentrifuged (Beckman L5-50 and L3-50) at a speed of 125,000 *g* for 24 h at 6°C. The lipoproteins were harvested by carefully pipetting the upper 1.5-ml fraction.

Chromatographic (HPLC) separations of lipoproteins

The apparatus consisted of a Waters Assoc. Model 204 liquid chromatograph, a Model 6000A pump, with a high-sensitivity noise filter in line, a Model 440 UV detector, and a WISP autosampler. This was coupled with a Perkin-Elmer Model Sigma 15 integrator for plotting and integration.

The 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.1 *M* sodium sulfate and 0.05% sodium azide was filtered through 0.45-μm Millipore filters and degassed. The 0.2 *M* Tris—acetate buffer, pH 7.0, containing 0.05% sodium azide was filtered and degassed prior to use. Initial separation of lipoproteins was performed using a gel permeation column TSK 4000 SW (Kratos, 600 × 7.5 mm). Later studies were conducted by using a TSK 4000 PW (600 × 7.5 mm) followed by a TSK 3000 PW (600 × 7.5 mm). Both column set-ups were preceded by the appropriate guard column TSK GSWP or GPWP (Kratos, 100 × 7.5 mm). Using the phosphate or Tris—acetate buffer at pH 7.0 with a flow-rate of 0.2 ml/min, proteins were detected by monitoring absorbance at 280 nm (attenuation of 3 using Perkin-Elmer Sigma 15 recorder—integrator) and a chart speed of 0.1 cm/min. Samples were applied automatically in a total volume of 150 μl equivalent to 50 μl serum or plasma. The autosampler was used for a 24-h operation.

RESULTS

HPLC separation of ultracentrifugally isolated standard lipoprotein fractions

Lipoprotein standards were isolated by sequential ultracentrifugation and were washed once by diluting with a potassium bromide solution of equivalent density and ultracentrifuging under similar conditions as described above for isolation of standard lipoproteins. These fractions gave a single peak when separated by HPLC (Fig. 1). VLDL had a retention time of 61.74 min (corresponding to an elution volume of 12.35 ml). LDL and HDL had retention times of 81.39 and 104.16 min, respectively. The HDL fraction had mainly HDL_2 and therefore, the retention time of 104.16 min corresponds to the HDL_2 fraction. The HDL fraction was contaminated with albumin (even after washing) as judged by SDS-PAGE [24] and, therefore, gave another HPLC peak corresponding to albumin (retention time of 115.18 min).

A number of other standards with different molecular weights were sep-

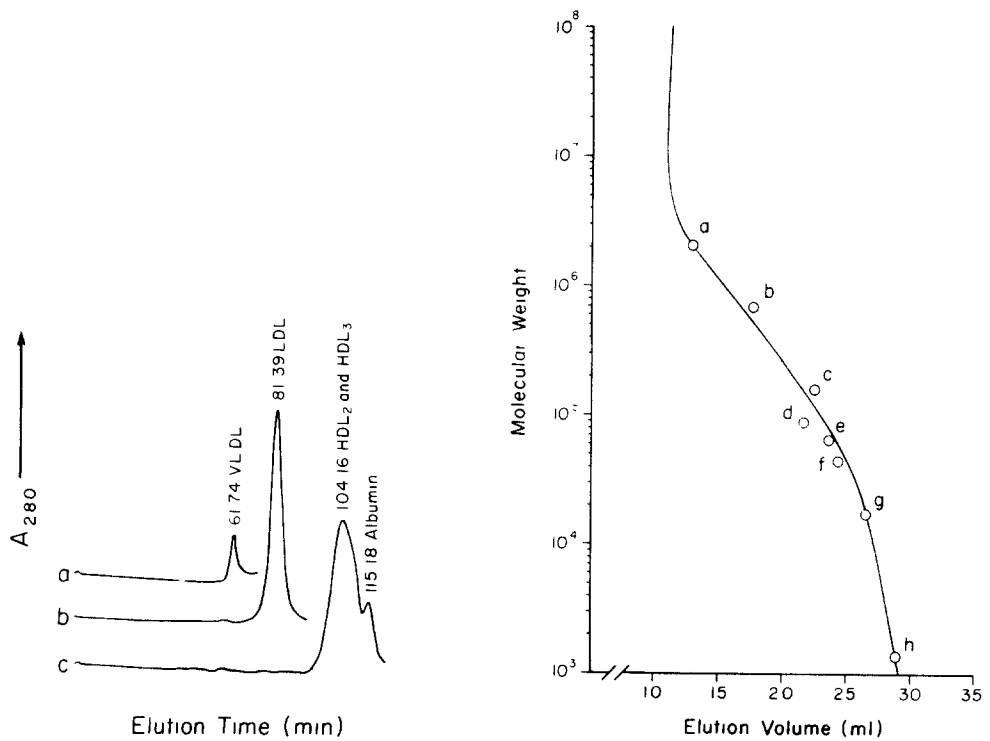

Fig. 1. HPLC elution patterns of the A_{280} for baboon lipoproteins isolated by sequential ultracentrifugation. Column, TSK 4000 SW (600 × 7.5 mm) + GSWP guard column (100 × 7.5 mm); eluent, 0.2 *M* Tris—acetate buffer, pH 7.0; flow-rate, 0.2 ml/min. (a) VLDL (density <1.006 g/ml); (b) LDL (density = 1.019—1.063 g/ml); and (c) HDL_2, HDL_3, and albumin (density = 1 063—1.21 g/ml).

Fig. 2. Relationship between molecular weight and elution volume (× 5 elution time). HPLC conditions were the same as Fig. 1. Reference compounds: (a) blue dextrin (2,000,000); (b) thyroglobulin (670,000); (c) gamma globulin (158,000); (d) transferrin (83,000); (e) baboon albumin (60,000); (f) ovalbumin (44,000); (g) myoglobulin (17,000); and (h) vitamin B_{12} (1350).

arated to determine the linear range of the TSK 4000 SW column. As shown in Fig. 2, the column gave a linear response between molecular weights $2 \cdot 10$ and $3.6 \cdot 10^4$ daltons corresponding to elution volumes between 12 and 25 ml. Elution volumes for plasma lipoproteins fell within the linear range.

Effect of phosphate buffer and Tris—acetate buffer on HPLC separation of lipoproteins

Initial studies were conducted using phosphate buffer. Under these conditions only 80—90 lipoprotein samples could be successfully separated. Afterwards, the column would retain most of the lipoproteins. Washing with organic solvents such as methanol, tetrahydrofuran, dimethylsulfoxide, and tetramethylurea did not regenerate the column. Smaller proteins (MW < 60,000), however, were still separated by this column.

To determine if a change in buffer would prolong the column life, we used the Tris—acetate buffer to separate the lipoproteins by HPLC. Use of the Tris—acetate buffer improved the resolution of lipoproteins and increased the column life span. Using the Tris—acetate buffer more than 300 samples were processed by a single column during the initial phases of this study. After the initial six months of this study (using twelve columns), the second group of columns (six) were not of the same quality as the earlier TSK 4000 SW columns. The absorption of lipoproteins occurred within the first 50—80 samples resulting in a very high cost per sample. At this point it was necessary to change to PW type columns as discussed by Carroll and Rudel [21]. A combination of TSK 4000 PW plus TSK 3000 PW gave adequate detection of the F° 9—28 lipoproteins. Again we were able to run at least 300 samples per column.

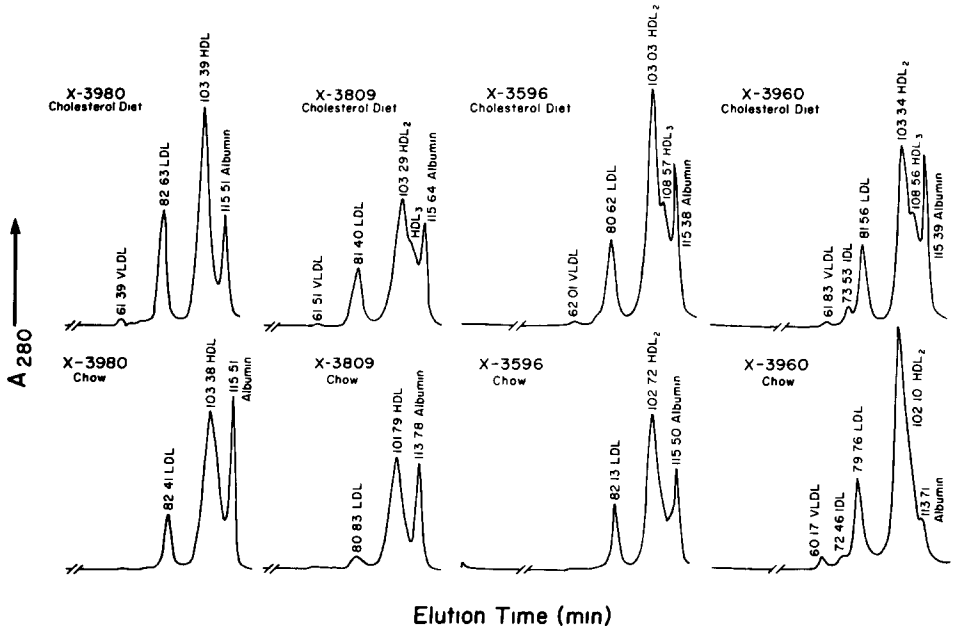

Fig. 3. Lipoprotein patterns from four representative animals, which do not show F° 9—28 lipoproteins on the chow (bottom) or atherogenic diet (top). Atherogenic diet has raised LDL and HDL levels.

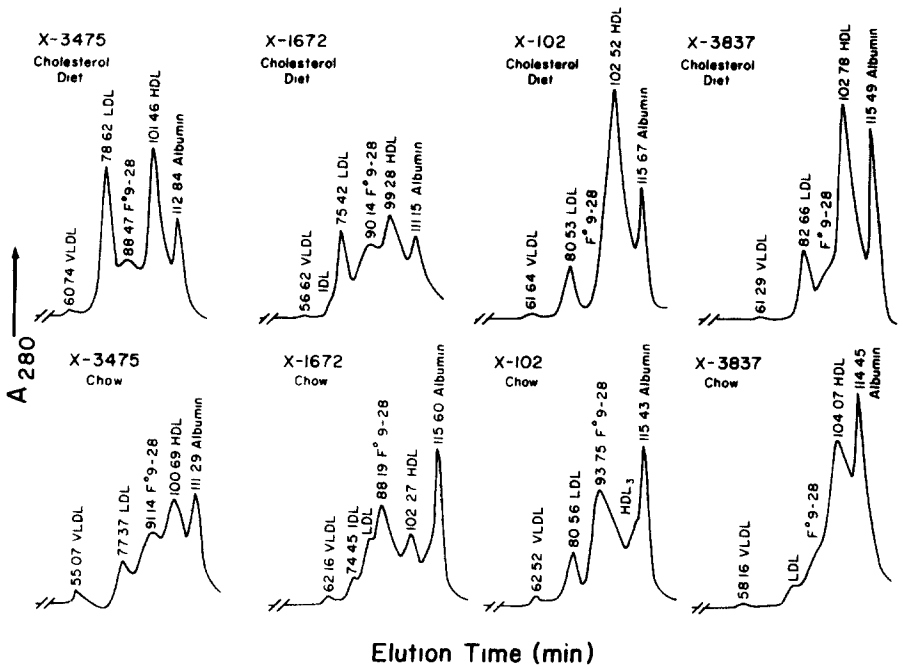

Fig. 4. Lipoprotein patterns from four representative animals, which show F° 9—28 lipoproteins on chow diet (bottom panel). F° 9—28 lipoproteins (elution time 88—91 min) appear either as a peak or as a shoulder to HDL towards LDL. Upon feeding atherogenic diet the F° 9—28 lipoproteins do not seem to be affected to a considerable extent (upper panel). LDL and HDL have been raised by feeding atherogenic diet as in case of animals without F° 9—28 lipoproteins.

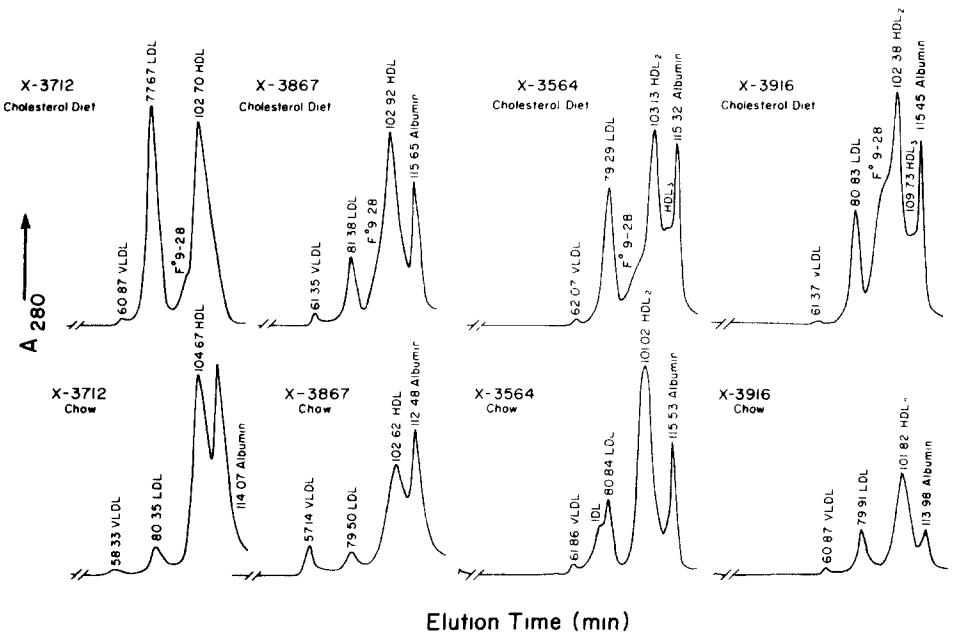

Fig. 5. Lipoprotein patterns from four animals that did not show high levels of F° 9—28 lipoproteins on chow diet (lower panel) but seem to induce these lipoproteins upon feeding atherogenic diet (upper panel).

Lipoprotein profiles of chow and atherogenic diet-fed animals

The majority of the animals on chow diet had lipoprotein profiles similar to those exhibited in Fig. 3. The major peak (A_{280}) corresponded to HDL_2. Varying amounts of LDL were present. VLDL was present in minute quantities, but was detectable in most animals. Since the lipoproteins (density <1.21 g/ml) were not washed, albumin was detectable in variable amounts. Upon cholesterol and saturated fat feeding their lipoprotein profiles changed. There was an increase in LDL and HDL with a distinct appearance of an HDL_3 fraction (elution time 108.19 min) in many animals. The VLDL concentration did not seem to change (Fig. 3).

As shown in Fig. 4, a distinct peak between LDL and HDL was detected in some animals which would correspond to the lipoprotein fraction between LDL and HDL_2 with a hydrated density F°_{120} between 9 and 28. These F° 9—28 lipoproteins were present in variable amounts on both chow and the atherogenic diet. Some animals on the other hand did not exhibit these F° 9—28 lipoproteins on the chow diet, but they seem to appear upon feeding an atherogenic diet (Fig. 5).

DISCUSSION

F° 9—28 lipoproteins are abnormal lipoproteins and were previously detected by analytical ultracentrifugation in some of the baboons fed a diet high in saturated fats and cholesterol [1]. The results of the present study demonstrate that gel permeation HPLC using either TSK 4000 SW or a combination of TSK 4000 PW and TSK 3000 PW columns can detect this lipoprotein and is suitable for screening a large population of pedigreed baboons. This technique is simple and rapid and therefore could be used three times (once on a chow diet and twice (seventh and eighth weeks) on an atherogenic diet) for 400—500 baboons. The use of a Tris—acetate buffer, pH 7.0, improved the resolution. The TSK 4000 SW columns, however, cannot be used for large numbers of samples, because they start absorbing lipoproteins. Carroll and Rudel [21] have similarly reported that these columns absorb lipoproteins. A combination of TSK 4000 PW and TSK 3000 PW, however, gave similar results and would, therefore, be of choice for similar studies. Albumin was seen as a contaminant in lipoproteins and gave a separate peak with a retention time of 115.8 min. The presence of an albumin peak, however, enabled us to calculate the relative retention of various lipoproteins for the new or other sets of columns. This relative retention time for lipoproteins did not change in the various columns used.

The comparison of plasma lipoprotein profiles of baboons indicated that F° 9—28 lipoproteins were not only present in some baboons on an atherogenic diet as observed earlier [1], but they were also present in some baboons on a low-cholesterol chow diet. This special class of lipoproteins was detected either as a distinct peak between LDL and HDL or as a distinct shoulder to the HDL_2 peak. The F° 9—28 lipoproteins were present in some baboons on both the chow and the atherogenic diet, but in certain cases it seems to be induced by feeding the atherogenic diet.

HDL_C, a lipoprotein induced upon cholesterol feeding [25] and Lp(a) lipo-

protein or sinking prebeta lipoprotein [26] in humans have been shown to occur between the LDL and HDL density region. Since Lp(a) has an apo-B-like protein as its major protein, it would be different from F° 9—28 lipoproteins which have apo-A-I and E as their major apoproteins. The F° 9—28 lipoproteins may, however, be similar to HDL_C. Further characterization of these abnormal lipoproteins in baboons will determine if this is the case. One major difference between HDL_C and F° 9—28 lipoproteins, however, is that HDL_C is induced upon cholesterol feeding, whereas F° 9—28 lipoproteins have been seen on a low-cholesterol, low-fat chow diet. The application of this technique has provided the means of phenotyping for the F° 9—28 lipoproteins. The collection of these fractions from preparative samples would provide material for lipid and apoprotein characterization of this lipoprotein. The metabolic basis and genetic inheritance of this lipoprotein are presently being investigated.

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