papers and notes on methodology

Rapid on-line determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography

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Abstract A high-performance gel chromatography (HPGC) system has been developed which allows the unattended on-line determination of lipoprotein cholesterol distribution (VLDL-C, LDL-C HDL-C), within 40 min, in microliter quantities of plasma using a single, relatively inexpensive column (Superose 6HR). The FAST cholesterol reagent (Sclavo) and a knitted PFTE Kratos reaction coil (Applied Biosystems) were found to provide optimal sensitivity, linearity, resolution, and dispersion characteristics. Validation is provided by comparison to target values for human quality control reference sera, and by comparing the values obtained by HPGC to the beta-quant method (LRC). The utility of the system is illustrated by comparing profiles from seven different species with normal or elevated plasma cholesterol concentrations. This technique allows rapid analysis of samples, regardless of species, without the use of precipitating agents or the ultracentrifuge. It could also be applied for the direct clinical determination of LDL-cholesterol—**Kieft, K.A., T. M. A. Bocan, and B. R. Krause**. Rapid on-line determination of cholesterol distribution among plasma lipoproteins after high-performance gel filtration chromatography. *J. Lipid Res.* 1991. **32:** 859–866.

Supplementary key words VLDL • LDL • HDL

In humans and experimental animals, cholesterol in plasma is transported in lipoprotein particles that differ in their hydrated density, net charge, and size. Numerous methods have been described that exploit one or more of these properties in order to separate plasma lipoproteins, such as sequential preparative ultracentrifugation, nondenaturing gradient gel electrophoresis, and paper or agarose electrophoresis. But these methods are very time-consuming, provide poor resolution, or are difficult to quantitate. Precipitation of apoB-containing lipoproteins in plasma by heparin-$MnCl_2$ or dextran sulfate-$MgCl_2$ provides a rapid estimation of HDL-cholesterol, but optimal conditions must be established separately for each animal species evaluated, and relatively large volumes of plasma are required.

Recently, Hara and Okazaki (1) have reviewed their extensive work on the separation of lipoproteins by high-performance liquid chromatography. In one application (2) they describe a combination of separation by HPLC and the on-line detection of cholesterol in the postcolumn effluent using an enzymatic reagent. This method would seem to be ideal for the rapid determination of cholesterol distribution in a large number of samples. However, the three large columns required for this application, namely two G3000SW and one G5000PW, are relatively expensive and have a short life span (3). In addition, the enzymatic reagent (and the chromogen) used by these investigators (Determiner TC "555," Kyowa Hakko Co., Tōkyo) is unavailable in the United States and contains high concentrations of costly enzymes. The present report describes the first evaluation of several readily available cholesterol reagents and reaction coils for the on-line determination of cholesterol distribution following high-performance gel chromatography (HPGC) using a single Superose 6HR 10/30 column. Optimal running conditions for this system allow the unattended determination of cholesterol distribution in plasma volumes of as little as 1 μl within 40 min. A preliminary report has appeared using this methodology for experimental rat studies (4), and here we provide both validation of the system and a further illustration of its broad utility by comparing profiles in humans to several species fed normal or cholesterol-containing diets.

Abbreviations: HPLC, high-performance liquid chromatography; HPGC, high-performance gel filtration chromatography; AAP, 4-aminoantipyrene; HDCBS, 2-hydroxy-3,5-dichlorobenzene sulfonic acid; C, cholesterol; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein.

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MATERIALS AND METHODS

Reagents and reference materials

Cholesterol oxidase (25 U/mg) and cholesterol esterase (26 U/mg) were purchased from Boehringer Mannheim (Indianapolis, IN). PIPES buffer, 4-aminoantipyrene (AAP), cholic acid (sodium salt), potassium chloride, Triton X-100, and horseradish peroxidase (P 8250) were from Sigma Chemical Company (St. Louis, MO). 2-Hydroxy-3,5-dichlorobenzene sulfonic acid (HDCBS, sodium salt) was obtained from Research Organics (Cleveland, OH). The commercially available cholesterol quantitation reagents were A-GENT Clinical Chemistry Cholesterol Reagent (Abbott Labs, North Chicago, IL), DMA Data-Zyme (Data Medical Associates, Inc., Arlington, TX), FAST Cholesterol (Sclavo Diagnostics, Wayne, NJ), and TRACE Reagent (Trace/America, Miami, FL). These cholesterol reagents were reconstituted in HPLC-grade water according to manufacturers' instructions, filtered through a 0.22-μm filter, and degassed immediately prior to use. Our "in-house" reagent, modified from Cooper et al. (5), was prepared by adding 50 ml of 60 mM HDCBS in 50 mM PIPES buffer, pH 6.9, to 50 ml of a solution containing 1% Triton X-100, 5.1 mM AAP, 6 mM cholic acid, and 200 mM potassium chloride in 50 mM PIPES buffer. Cholesterol oxidase (12.5 U), cholesterol esterase (78 U), and peroxidase (1250 U) were then added and the reagent was filtered and degassed. The enzyme concentrations for the other cholesterol reagents are listed in **Table 1**. The in-house reagent must by prepared daily, but the Sclavo reagent is stable for 45 days, the Trace and DMA reagents for 30 days, and the Abbott reagent for 7 days at 4°C. Lipoprotein cholesterol quality control materials, consisting of frozen aliquots of fresh, pooled human serum with accurate target values, were obtained from Solomon Park Research Laboratories (Kirkland, WA). Plasma samples (EDTA) for determining precision (CVs) were obtained from fasted healthy volunteers recruited by the Warner-Lambert/Parke-Davis Community Clinic.

HPGC system

The HPGC system consisted of a Waters 712 Wisp (automatic sample injection system), two Waters M-6000A solvent pumps, a Waters 440 absorbance detector, and a Waters Model 680 Automated Gradient Controller (Waters Chromatography Division, Millipore Corporation, Milford, MA). The column effluent was combined with the enzymatic reagent through a zero dead volume T-connector. The enzymatic cholesterol reaction was carried out in the various reaction coils in a Precision thermostatted water bath at 37°C (**Fig. 1**). Data were integrated using a Hitachi D-2000 Chromato-Integrator (EM Science, Cherry Hill, NJ). Lipoprotein separations were performed on a single Superose 6HR FPLC column (10 × 300 mm), preceded by a column prefilter (Pharmacia LKB, Piscataway, NJ). Elution buffer consisted of 0.15 M NaCl, pH 7.0, containing sodium azide (0.02%). Flow rates for the main pump and the cholesterol reagent pump were 0.5 ml/min and 0.2 ml/min, respectively. The column was calibrated using human lipoprotein standards prepared by sequential ultracentrifugation (6). The recovery of human low density lipoproteins (LDL) was determined both by mass measurements and by recovery of radioactivity after injection of LDL iodinated by the method of McFarlane (7) as modified by Bilheimer, Eisenberg, and Levy (8). The various peaks in the cholesterol profiles are designated VLDL, LDL, and HDL for simplicity, even though it is clear that the separation is determined primarily by the size of the lipoproteins, and that in some instances (e.g., dyslipidemic states, see below) the term LDL may include particles that

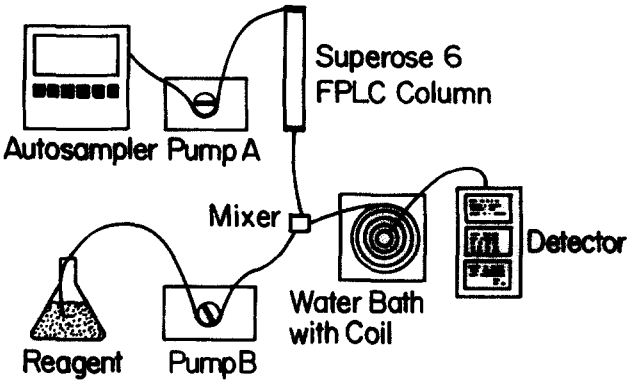

Fig. 1. Configuration of HGPC components.

TABLE 1. Comparison of cholesterol reagents[a]

Enzyme	In-House	A-GENT	FAST	TRACE	DMA	Hara
Cholesterol esterase, U/l, (origin)[b]	780 (B)	117 (M)	>250 (B)	200 (M)	1000 (B,M)	1000 (?)
Cholesterol oxidase, U/l	125	167	>250	150	400	1875
Peroxidase, U/ml	25	28	>4	2	6	6.7
4-Aminoantipyrine, mM	2.5	0.8	0.3	0.5	0.2	1.0
HDCBS, mM	29		8			

[a]See Materials and Methods for manufacturer identification; Hara refers to reference 1.
[b]Letters in parentheses refer to origin: B, bacterial; M, mammalian.

would float at 1.006–1.019 g/ml or IDL (9). When several different reactors were compared, both resolution and dispersion of the peaks were calculated (**Table 2**). Resolution or separation of two adjacent bands (VLDL and LDL) was measured in terms of the resolution function R_s, where $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ or the difference in retention times $(t_2 - t_1)$ for the two bands, divided by the average baseline band width in time units (10). Peak dispersion was measured by calculating H, the height equivalent of a theoretical plate, as $H = (L/5.54)(w_h/t^2)$, where L is the tube length in cm, w_h is the peak width at half height, and t is the delay time from injection to detection of the peak maximum (w_h and t are measured in sec) (11). Cholesterol profiles in both normolipidemic and dyslipidemic states (see below) were described by both percentage distribution of cholesterol and retention times (see Table 5). Validation of the HPGC system was obtained by comparing HPGC values to the target values for human reference serum pools (see Table 4), and also by comparison to values for lipoprotein cholesterol using the "beta-quant" method (12). In this method VLDL is isolated directly by a single ultracentrifugal spin. LDL is obtained by subtracting HDL-C, determined by precipitation of a separate aliquot of plasma, from the cholesterol in the bottom fraction. This method, or variations thereof, is used clinically to estimate lipoprotein concentrations (e.g., LRC Program). For the cholesterol measurements of these fractions we used the Abbott VP Analyzer with the FAST reagent.

Animals

Yucatan micropigs (10–12 kg) were meal-fed a normal chow diet (Purina No. 5080) or a purified diet for 2 weeks that contained 20% soy protein, 24% sucrose, 2% cholesterol, 8% coconut oil and 8% peanut oil (Research Diets, Inc., New Brunswick, NJ). Golden Syrian hamsters (male, 110–120 g) were fed chow (Purina No. 5012) or a purified diet containing 20% casein, 36.5% sucrose, 24% corn starch, 10% coconut oil, 1% corn oil, 0.06% cholesterol, 8% cellulose fiber (Avicel PH101), and AIN-76A vitamin and salt mixes, again for 2 weeks (Research Diets, Inc.). Rabbits (New Zealand males, 1.2–1.5 kg) were meal-fed normal chow (Purina No. 5321) or chow supplemented with 0.5% cholesterol, 3% coconut oil, and 3% peanut oil for 17 weeks, as previously described (13). Male Sprague-Dawley rats (200–225 g) were fed either ground rat chow (Purina Lab Chow No. 5002) or chow supplemented with 5.5% peanut oil, 1% cholesterol, and 0.5% cholic acid for 1 week (14). Guinea pigs (Hartley males) were fed normal chow (Purina No. 5025) or chow supplemented with 0.5% cholesterol and 15% lard for 2 weeks. The guinea pigs on normal chow were retired breeders (>800 g), whereas the cholesterol-fed animals were virgin weighing approximately 300 g. Dogs (male Beagles, 10–12 kg) received normal dog food (Purina No. 5006) or food supplemented with 0.2% cholic acid, 1% cholesterol, and 5.5% lard for 6–8 weeks. All blood samples were taken from nonfasted animals (except micropigs which were fasted) and placed into tubes containing EDTA (1.5 mg/ml). Plasma was stored at 4°C and used within 1 week after the animal was killed.

RESULTS AND DISCUSSION

Evaluation of cholesterol reagents

A pool of plasma from cholesterol-fed dogs was used to determine the linearity and sensitivity of the various cholesterol reagents (**Fig. 2**). The cholesterol concentration of this pool was 400 mg/dl. A 20-m coil was used for these comparisons since it possessed the longest reaction time (5.8 min). Reagents that generally require reaction times longer than 5.8 min were found to be unsuitable for the present HPGC application. The DMA, TRACE and A-GENT reagents needed longer reaction times to develop sufficient colored product for detection under these conditions. One possible explanation for the low response of the A-GENT and TRACE reagents could be the relatively low concentration of cholesterol esterase,

TABLE 2. Characteristics of postcolumm reactors

Reactor[a]	Type	Material	Length	Resolution	Dispersion
			m	*sec*	*cm*
A	coiled	SS	20.0	1.026	5.427
B	coiled	SS	15.24	1.039	2.237
C	knitted	PTFE	13.1	1.222	0.578
D	knitted	PTFE	6.1	1.209	0.999
E	knitted	PTFE	12.2	1.158	1.038
F	knitted	PTFE	6.1	1.137	0.476

Peak dispersion and resolution were calculated as described (10, 11). Reactors A and B were custom-ordered from Bodman Chemicals (Media, PA). Reactors C, D, and E are from Kratos/Applied Biosystems (Ramsey, NJ), Waters (Milford, MA), and Supelco (Bellefonte, PA). Reactor F is a two-segment version of the four-segment Supelco reactor; SS, stainless steel; PTFE, Teflon.

[a]Designations are from the panels in Fig. 3.

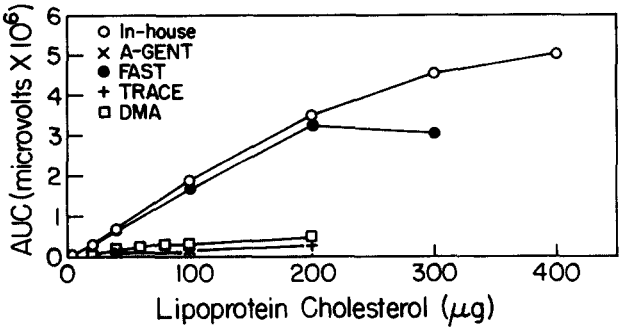

Fig. 2. Comparison of enzymatic cholesterol reagents. See Methods for complete descriptions of these reagents. Sample: plasma from cholesterol-fed dogs (cholesterol concentration = 400 mg/dl): loaded volume, 1–100 μl; coil, stainless steel, 20 m × 0.508 mm i.d.; AUC, area under the curve. For the FAST and in-house reagents r = 0.9996 and 0.9988, respectively, from 4 to 200 μg cholesterol.

whereas with the DMA product the need for a long reaction time must be due to other undefined factors such as surfactant or HDCBS concentration. In contrast, with the in-house and FAST reagents, the colored reaction product, a quinoneimine, developed quickly and was linearly related to the lipoprotein-cholesterol concentration over a range of 4 to 200 μg. The source of the cholesterol esterase enzyme may also be a factor, as shown by others (15), since A-GENT and TRACE utilize mammalian and the in-house and FAST utilize bacterial enzymes. The mammalian enzyme may not hydrolyze all of the cholesteryl esters in this plasma from cholesterol-fed dogs. This would not explain, however, the low sensitivity of the DMA reagent which contains both mammalian and bacterial esterase. A unique advantage of the in-house reagent is the ability to measure lipoprotein-free cholesterol content in a sequential plasma sample by the omission of cholesterol esterase. The quantitation of free and ester content could have utility in lipoprotein compositional analysis. But the use of the FAST reagent, as in the following coil and species comparisons, requires no preparation except for the addition of water.

Reaction coil comparisons

The Superose 6HR column cannot withstand the backpressure developed by the small internal diameter of the reaction coil (0.25 mm) previously described by Okazaki, Ohno, and Hara (2). A larger internal diameter of 0.508 mm (0.02 in) was found to be the minimum that could be used. Therefore, after identification of a suitable, relatively inexpensive cholesterol reagent, a number of reaction coils (postcolumn reactors) with this minimum diameter were examined in order to optimize the separation. Both coiled and knitted reactors were compared. The extracolumn band broadening characteristics of open tubular coiled reactors due to a parabolic flow profile is well documented (16). Knitted reactors are commonly used to break up the parabolic profile and to induce a secondary flow pattern that minimizes band broadening. The FAST cholesterol reagent and plasma from euthyroid cholesterol-fed dogs were used for these coil comparisons (**Fig. 3**). The lengths for both the Waters and the two-segment Supelco knitted coils (6.1 m) were not sufficient for reaction completion of HDL-sized lipoproteins, as evidenced by the formation of incomplete peaks, typical of excess lipoprotein cholesterol in the reaction mixture (Fig. 3D and 3F). When the resolution (separation of two adjoining peaks, 10) and band dispersion (11) were calculated as a measure of performance of these reactors (Table 2), the knitted reactors had consistently higher peak resolution and lower dispersion as expected from their geometry (Table 2). The Applied Biosystems coil (Fig. 3C) was found to have the best separation qualities and reaction length for this system and has been incorporated into the routine analysis of samples in our laboratory.

Recovery, precision, and validation

Cholesterol was recovered quantitatively when a known mass of lipoprotein-cholesterol (3.44 mg human LDL) was applied and subsequently analyzed in the separate lipoprotein fractions using the enzymatic method (recovery = 108%). In addition to mass measurements, recovery was also determined using labeled human LDL. Specifically, when human plasma was spiked with fresh

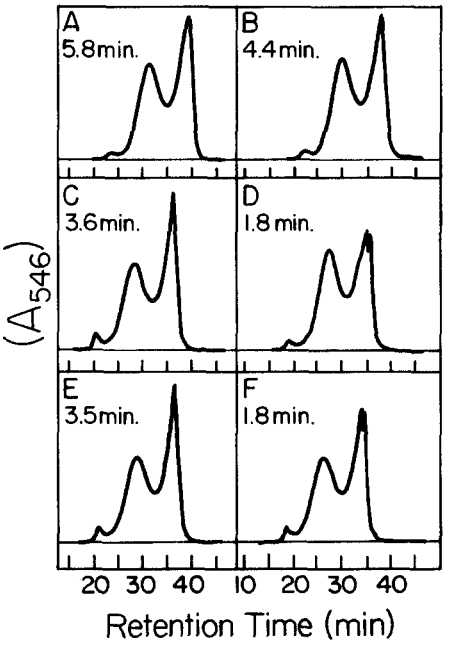

Fig. 3. Comparison of cholesterol distribution profiles using different reaction coils. The numbers in each panel are the reaction times in minutes. Other characteristics are listed in Table 2. Sample: plasma from cholesterol-fed dogs: sample size, 25 μl; reagent, FAST cholesterol reagent (Sclavo).

iodinated LDL (6,300 cpm), 93% of the injected radioactivity eluted from the column, of which 89% was in the LDL region. These recovery figures compare favorably with those of Ha and Barter (17) who reported 90% recovery of cholesterol mass using Superose 6B in a larger column (56 cm × 1.6 cm). Precision was determined by calculating both intra- (ten samples run on the same day) and inter- (ten samples run on consecutive days) assay coefficients of variation. The corresponding values were 1.4% and 2.2%, respectively, using human plasma. Validation of the HPGC method was demonstrated in three ways. First, when ultracentrifugally isolated human VLDL, LDL, and HDL were separately analyzed, they eluted with identical retention times as for whole plasma. Secondly, the values obtained by HPGC were compared to the beta-quant method for estimating VLDL-C, LDL-C, and HDL-C using plasma samples from ten healthy volunteers. In this analysis, the percentage distributions (**Table 3**) were not significantly different using these two techniques, and the mean values for total cholesterol were also comparable. Absolute values (mg/dl) are not provided in the table since in our hands the recovery for the beta-quant method, which involves harvesting and analysis of the d < 1.006 g/ml lipoproteins, is only 80%. Finally, we compared the lipoprotein cholesterol target values for human serum reference pools to values obtained by HPGC (**Table 4**). All values for LDL and HDL (mg/dl) fell within the acceptable ranges calculated by the supplier (Solomon Park Research Laboratories). During the course of these validation experiments we also compared the HDL values for normal rat plasma obtained by dextran sulfate precipitation to those obtained by HPGC. We found that HPGC resulted in significantly higher HDL values compared to dextran sulfate precipitation (70% cholesterol in HDL vs. 60% for dextran sulfate, n = 10). This is most likely due to the greater amount of apoE-containing HDL in this species, which is precipitated by dextran (18).

TABLE 3. Comparison of beta-quant[a] method with HPGC

	VLDL		LDL		HDL	
Subject	B-Q	HPGC	B-Q	HPGC	B-Q	HPGC
			% distribution			
1	8.2	9.1	66.4	64.4	25.4	26.6
2	4.6	5.9	75.0	73.4	20.4	20.7
3	5.5	6.9	73.8	70.0	20.7	23.0
4	1.7	2.5	69.5	67.0	28.8	30.5
5	7.2	4.1	68.9	68.2	23.9	27.7
6	11.7	11.9	67.9	65.2	20.3	22.9
7	0.2	2.1	67.7	64.3	32.1	33.7
8	3.9	5.1	81.7	78.4	14.4	16.5
9	0.7	1.6	64.0	60.7	35.3	37.7
Mean	4.9	5.5	70.5	67.9	24.6	26.6
± SEM	1.3	1.2	1.8	1.8	2.2	2.0
r	0.9225		0.9830		0.9889	

The mean plasma total cholesterol concentration was 194 ± 14 using the Abbott VP Analyzer and 207 ± 14 for the HPGC (NS, r = 0.9980). For both methods, the FAST reagent was used.

[a]"Beta-quant" is a method for lipoprotein cholesterol determination in which VLDL-C is removed by ultracentrifugation (d < 1.006 g/ml), and LDL is determined by subtracting the cholesterol in dextran sulfate supernatants (HDL-C) from the cholesterol in the d > 1.006 g/ml (bottom) fraction (12).

TABLE 4. Comparison of lipoprotein cholesterol quality control target values to values obtained by HPGC

Pool[a]	VLDL	LDL	HDL
		mg/dl	
1			
Target[b]	13.4	97.1	72.5
Range	12.7-14.1	92.2-101.0	65.3-79.8
HPGC	12.4	93.7	75.0
HPGC	11.9	92.0	74.0
HPGC	12.6	99.8	78.0
HPGC	11.5	93.7	72.0
2			
Target	25.1	157.9	61.0
Range	23.8-26.3	150.0-165.8	54.9-67.1
HPGC	25.2	153.9	58.0
HPGC	21.7	158.9	58.0
HPGC	21.9	161.3	63.0
HPGC	20.5	153.5	57.0

[a]The plasma total cholesterol values for these human serum pools were 183 mg/dl (range: 174-192 mg/dl) for pool 1 and 244 mg/dl (range: 231-256 mg/dl) for pool 2. Values obtained by HPGC fell into these ranges.

[b]Calculated from data supplied by Solomon Park Research Laboratories (Kirkland, WA), where LDL-C = total - HDL-C - VLDL-C (TG/5).

Applicability

The utility of the HPGC system is illustrated in **Fig. 4** by comparing the cholesterol distribution profiles in human subjects to those of six different animal species fed normal chow or cholesterol-containing diets. In some cases purified diets are compared to nonpurified diets within a given species, so differences are not due solely to the effect of dietary cholesterol. Moreover, a single animal is analyzed in each panel and thus any apparent differences would require confirmation in a larger series. Nonetheless, applicability of the HPGC system can be clearly indicated using a variety of species and diets. For example, using HPGC we find that the micropig normally transports equal amounts of cholesterol in LDL and HDL, but most cholesterol resides in LDL (78%) when these animals are fed a purified atherogenic diet. Unlike many other species, hamsters develop marked hypertriglyceridemia in response to fat/cholesterol ingestion; HDL-C levels generally remain unchanged while VLDL increases. In our hands LDL constitutes only between 20 and 30% of total cholesterol in hamsters, regardless of diet. In the chow-fed rabbit profile in Fig. 4, 60% of

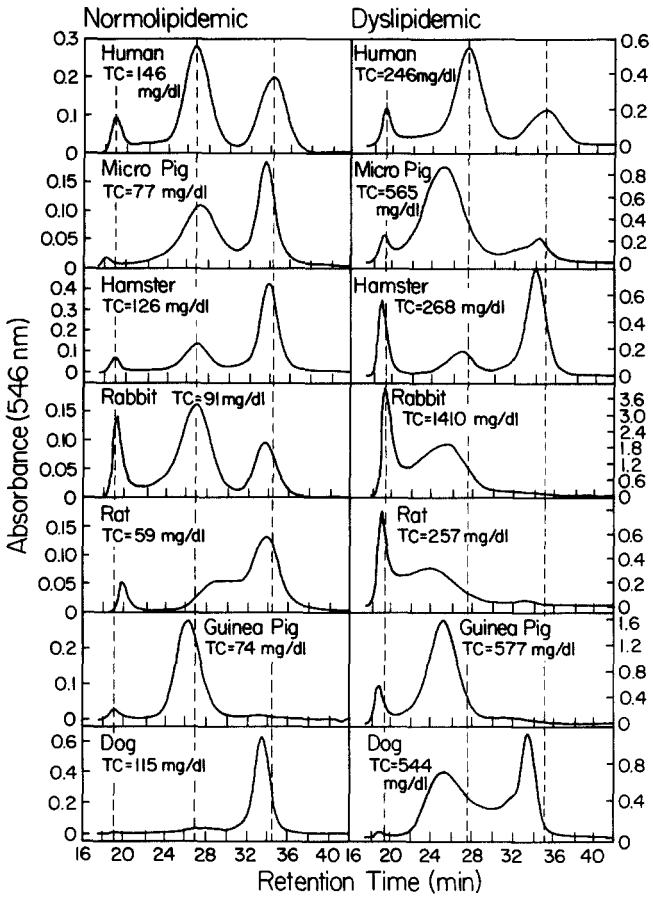

Fig. 4. Cholesterol distribution profiles for seven species with normal (normolipidemic) or abnormal (dyslipidemic) plasma total cholesterol concentrations. Details for the animal models are in the Methods and Materials section. Plasma total cholesterol concentrations (TC), derived by calibration using the Solomon Labs human reference pools (Table 4), are indicated for each profile. The vertical, dashed lines mark the elution peaks for human VLDL, LDL, and HDL. The precise %-distributions and retention times for these profiles are provided in Table 5. Coil: 13 m, knitted PTFE (coil C in Table 2); reagent: FAST (Sclavo), Sample volumes: 5–40 μl.

cholesterol resided in LDL, with the remainder in VLDL (16%) and HDL (24%) (**Table 5**). Upon cholesterol feeding, the percentage of VLDL increased to 33% and essentially no HDL was found. Data in the literature are variable as to the cholesterol distribution in normal rabbits, with some investigators also reporting more LDL than HDL (19, 20), and others finding more HDL than LDL (21, 22), or equal amounts of both (23–25). With cholesterol feeding, however, an increase in VLDL-cholesterol is uniformly reported, with the extreme case being a complete loss of cholesterol in LDL and HDL (26). The HPGC data for rats are in agreement with that reported previously using sequential ultracentrifugation for both the chow (27) and cholesterol (28) diets, the latter resulting in much larger percentages of VLDL and the most dramatic change in the percentage of HDL of all species examined. Guinea pigs have essentially only LDL regardless of diet and, like the cholesterol-fed hamster, the percentage of HDL does not change with cholesterol feeding. Finally, in the chow-fed dog illustrated in Fig. 4, essentially all cholesterol was found in HDL (88%). Others have reported that about 80–85% of plasma cholesterol is normally found in HDL in this species (29, 30). Unlike the profile in hypothyroid/cholesterol-fed dogs in which most cholesterol is transported in VLDL (31), the euthyroid dogs fed cholesterol and fat have very little VLDL and variable amounts of LDL depending upon the degree of hypercholesterolemia. Although not studied in detail here, we have found that in rabbits and guinea pigs, which have cholesteryl ester transfer protein, the length of time that the samples are in an autosampler at room temperature alters the subsequent cholesterol disribution profile. Thus, it is very important that samples from such species be measured the same day using single injections or by using a refrigerated autosampler.

Peak retention times for the various species are also listed in Table 5. As with conventional agarose column chromatography (9, 31), these data can be used to estimate and compare the relative sizes of the lipoprotein particles. With human plasma from normolipidemic volunteers, we observed peak retention times of 19, 27, and 35 min for VLDL, LDL, and HDL, respectively. In general, these values are similar to those found in the other species, although some differences are apparent. For

TABLE 5. Percentage distribution (%) and retention times (RT) for profiles of chow- and cholesterol-fed animals

	VLDL		LDL		HDL	
Species	%	RT	%	RT	%	RT
		min		*min*		*min*
Human						
N	7	19	55	27	38	35
D	9	19	65	28	26	35
Micropig						
N	2	18	51	27	47	34
D	6	19	78	25	16	34
Hamster						
N	6	19	29	27	65	34
D	21	19	19	27	60	34
Rabbit						
N	16	19	60	27	24	34
D	33	20	62	25	5	30
Rat						
N	8	20	33	32	59	34
D	41	19	55	24	4	33
Guinea pig						
N	4	19	91	26	5	33
D	11	19	84	25	5	31
Dog						
N	1	18	11	27	88	34
D	1	18	54	25	45	34

Data are from the profiles depicted in Fig. 4; N, normolipidemic; D, dyslipidemic.

example, micropig VLDL appear larger and rat VLDL appear smaller compared to human VLDL under normolipidemic conditions. In addition, all species examined seem to have larger HDL compared to humans. In dyslipidemic states, there is an apparent increase in the size of LDL in most species compared to the LDL found under normal conditions, and the LDL in dog, guinea pig, micropig, and rabbit appear of similar size (RT = 25 min) compared to hamster or man. It is likely that the IDL reported earlier in cholesterol-fed rats (28, 32) and by others in cholesterol-fed rabbits (20, 23) using ultracentrifugation constitute a major portion of the "LDL" identified by HPGC.

In summary, the combined results demonstrate that the described single-column HPGC method with on-line cholesterol monitoring can rapidly determine cholesterol distributions quantitatively on microliter quantities of whole plasma using readily available reagents and components. We find the HPGC system to be much less labor intensive, less costly, and more reliable (i.e., better recovery) compared to methods that utilize ultracentrifugation, even compared to single-spin gradient ultracentrifugation using vertical rotors. The utility of this system is demonstrated by observing comparative differences in profiles among animal species, and the effect of various cholesterol-containing diets. More recently (33) we have been using the Beckman System Gold Chromatography System which has micro-flow pump heads better suited for the low back pressures (<215 psi) required for Superose HR columns. This newer system also is computer-controlled, making data acquisition and analysis automatic. Using standard curve calibration data, peak area can be easily converted to mg/dl. With either the Waters or Beckman system, the postcolumn lines could also be split to determine the distribution of other lipoprotein constituents (e.g., triglycerides, phospholipids) (1) or configured to include an on-line radioisotope detector for calculating specific activities. Although more rigorous validation may be required, this system could potentially have important clinical application since currently LDL-cholesterol is only estimated indirectly in most clinical laboratories.

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