USAFSAM-TR-90-12

LONG-TERM RISK FUNCTION EVALUATION: BIOCHEMICAL

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July 1990

Final Report for Period 1976 - 1990

USAF SCHOOL OF AEROSPACE MEDICINE
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NOTICES

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution is unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S): USAFSAM-TR-90-12	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: USAF School of Aerospace Medicine; 6b OFFICE SYMBOL (If applicable): USAFSAM/NGIS	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code): Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION: USAF School of Aerospace Medicine; 8b OFFICE SYMBOL (If applicable): USAFSAM/NGIS	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c ADDRESS (City, State, and ZIP Code): Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO 62202F; PROJECT NO 7755; TASK NO 25; WORK UNIT ACCESSION NO 04

11 TITLE (Include Security Classification): Long-Term Risk Function Evaluation: Biochemical

12 PERSONAL AUTHOR(S): Clark, Dale A.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM 1976 TO 1990	1990, July	20

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	01	
06	15	

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
HDL Cholesterol; HDL-C Quality Control; Lipoprotein composition; Lipoprotein titration

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

A method of determining the "true" level of HDL-cholesterol in serum was developed. The serum lipoproteins were titrated by adding increasing amounts of lipoprotein precipitant, measuring the cholesterol content in the supernate, and monitoring the lipoprotein content of the supernate by agarose gel electrophoresis. For quality control of routine HDL-cholesterol measurements, serum pools proved highly useful. A computer program that printed out values obtained for total cholesterol, HDL-cholesterol, and triglyceride levels during the last 10 laboratory runs, plus statistical information on the levels and the ratios between those levels, was a useful tool in identifying sources of error and was a strong motivating factor in technician morale. Various methods were used separately and in combination in unsuccessful efforts to develop a routine procedure for measuring the composition of serum lipoproteins. Density gradient centrifugation, agarose gel lipoprotein electrophoresis, and various chromatographic methods were used. The latter included size exclusion, affinity binding, HPLC, and antibody binding. Removal of the bulk of unwanted proteins was
(Cont'd)

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL: Dale A. Clark	22b TELEPHONE (Include Area Code): (512) 536-3127; 22c OFFICE SYMBOL: USAFSAM/NGIS

19. ABSTRACT (Continued)

easily accomplished, but the small amounts of albumin and globulins that remained prevented reliable measurements of lipoprotein composition. Specific antibodies removed not only their target proteins, but also significant amounts of lipoproteins. The identity of the remaining lipoproteins was therefore uncertain, as was the relationship of their (modified?) composition to the original composition of the serum lipoproteins. This problem requires further investigation before its solution is achieved.

LONG-TERM RISK FUNCTION EVALUATION: BIOCHEMICAL

INTRODUCTION

Objective: To determine whether selected biochemical factors (HDL cholesterol, lecithin:cholesterol acyltransferase activity, fatty acid composition of serum phospholipids, related biochemical substances) are significantly associated with coronary artery diseases as diagnosed by angiography.

Approach: Measure levels of the biochemical indicators in patients before and after heart catheterization and correlate the levels with the findings of the catheterization.

Modification of Objective: Lecithin:cholesterol acyltransferase (LCAT) activity (1) was measured in serum from several patients until experience with the method gave good agreement between duplicate measurements. However, differences in serum levels of various lipoproteins (2), free cholesterol (3), or phospholipids, or in the fatty acid composition of the phospholipids--one or several of these factors--appeared to affect significantly the measured activity of LCAT. Considerable research appeared necessary to establish a measurement protocol that would give meaningful physiological information from routine serum samples. Pursuit of this part of the original objective was therefore set aside pending further developments in the methodology.

The fatty acid composition of serum phospholipid fatty acids was determined in several patients by gas chromatography (4) using an instrument with limited sensitivity. Since this work was being duplicated by another investigator using a gas chromatograph with greatly improved sensitivity, this part of the original objective was abandoned.

MEASUREMENT OF SERUM HIGH-DENSITY LIPOPROTEIN CHOLESTEROL (HDL-C) LEVELS

Origination of a Method to Assess the Accuracy of Serum High-Density Lipoprotein Cholesterol (HDL-C) Levels by Titration of Serum with Increasing Amounts of Lipoprotein Precipitant

Reports that the average HDL-C level was lower in persons with coronary artery disease (CAD) than in normal persons (5,6,7) made it urgent to measure HDL-C levels in patients being evaluated for CAD at the USAF School of Aerospace Medicine (USAFSAM). The reported methods for measuring HDL-C were therefore explored. Laboratory determinations of HDL-C (8) by precipitation of the very low-density lipoproteins (VLDL) and the low-density lipoproteins (LDL) are based on the work of Burstein et al. (9), who showed that the combination of certain polyanions and divalent cations would precipitate serum lipoproteins. Since the VLDL and LDL precipitate before the HDL, it should be possible to add an amount of precipitant that would cause all the VLDL and LDL, but none of the HDL, to precipitate. The cholesterol concentration

remaining in the filtrate (or supernate) would then be the true HDL-C level. The extent to which this ideal situation can be approached determines the accuracy of the HDL-C measurement.

In actual practice, there is real danger that some of the HDL may fail to precipitate, causing the measured HDL-C level to be spuriously high. Conversely, if too much precipitant is added for the levels of lipoproteins present in the sample being analyzed, some of the HDL may be precipitated, causing the measured HDL-C level to be falsely low. The HDL fraction can be separated from the other lipoproteins by increasing the density of the serum and centrifuging the solution at high speed. However, this procedure is not suitable for routine clinical laboratory use.

In searching for an alternate way to establish the true HDL-C level in a serum sample, we originated a method of titrating the serum lipoproteins. To a series of tubes, each containing 1 ml of the serum sample, we added an increasing amount of precipitant as we progressed through the series of tubes (Fig. 1).

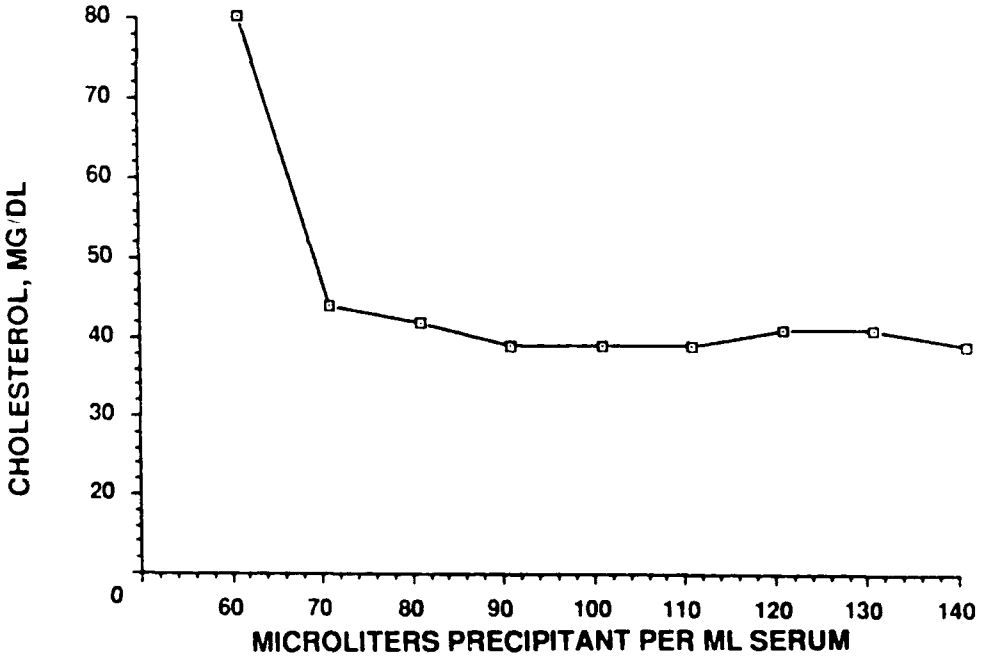

Figure 1. Titration of serum with lipoprotein precipitant (phosphotungstate $MgCl_2$). To each of a series of tubes containing 1.0 ml human serum, a volume of precipitant between 0 and 140 µl was added. To make the volume the same in all tubes, to each tube was added a volume of saline solution equal to 140 µl minus the volume of the precipitant that had been added. After precipitation, centrifugation, and careful decantation of the supernate, the concentration of cholesterol in the supernate from each tube was measured. For each tube in the series, that concentration and the corresponding volume of added precipitant are plotted on the Y and X axes, respectively.

After centrifuging down the precipitate and decanting the supernate in each tube, we measured the cholesterol level and analyzed the lipoprotein content of each supernate by agarose gel lipoprotein electrophoresis. When the cholesterol level measured in the supernate was plotted against the amount of precipitant added to that tube, the resulting curve first decreased steeply, plateaued, then fell off slowly as the amount of added precipitant increased from zero to the highest amount. When the intensity of the VLDL and LDL bands was similarly plotted, they fell steeply and disappeared at the start of the plateau region. The HDL band, however, appeared constant through all the tubes until the end of the plateau region where the cholesterol level began to fall. At that point, the intensity of the HDL band on the electrophorograms appeared to decrease with increasing amounts of added precipitant. These observations made it clear that the level of cholesterol measured in the plateau region of the curve was the true HDL-C level; the VLDL and LDL had precipitated, but the HDL was still present at its initial level. This procedure was published as SAM-TR-81-31 (10).

Development of a Routine Method for HDL-C Determinations

Because of the wide plateau region seen with titration studies using a phosphotungstate-$MgCl_2$ solution as the precipitant, the phosphotungstate method was chosen for routine in-house laboratory use. The titration procedure was used to establish the correct volume of precipitant required per ml of serum to most closely approach the ideal of precipitating all of the VLDL and LDL, but none of the HDL. The volume of precipitant used was only 0.1 ml per ml of serum instead of the 0.125 ml used in the original procedure (11). Our titration studies showed that the latter volume was too large for many serum samples. The 0.125 ml precipitant per ml serum often caused precipitation of some HDL and gave a low value for the HDL-C level (8). Further in-house studies resulted in fine-tuning of the pH of the reagent and of the temperature protocol for various steps in the procedures (12). The method was adapted to use only 0.5 ml volume of serum (13). Various aspects of these studies were discussed in papers presented at meetings of the Society of the Armed Forces Medical Laboratory Scientists (14) and the Aerospace Medical Association (15). The result of all these studies was an in-house capability for HDL-C determinations optimized for accuracy and repeatability when applied to routine serum samples from pilots evaluated by the Clinical Sciences Division at the USAFSAM.

Results of Use of the HDL-C Method

Application of the HDL-C method to serum of patients being evaluated for CAD by coronary angiography established the power of HDL-C level to predict risk of CAD in this patient population. One group of 572 patients, evaluated by heart catheterization, had a CAD prevalence rate of 14%. In this group (16), a ratio of total cholesterol/HDL-C having a value greater than 6.0 had a sensitivity of 86%, a specificity of 96%, and a predictive value of 78%.

Quality Control of HDL-C Determinations

Experience with various serum samples led to the recognition of pitfalls in the laboratory methods for measuring HDL-C. All of the common methods (heparin-$MnCl_2$, dextran sulfate, or phosphotungstate-$MgCl_2$) sometimes gave

erroneous results when the lipoprotein content of the serum samples was aberrant. The heparin-$MnCl_2$ method was most subject to these errors; the dextran sulfate and the phosphotungstate-$MgCl_2$ methods, in that order, were less subject to these errors. Some examples of problems with serum samples containing aberrant lipoproteins were published (15) to alert flight surgeons and clinical laboratory officers.

Many USAF clinical laboratories continued to have problems with HDL-C determinations. One such laboratory requested our assistance because their HDL-C levels were so low that the medical staff would not believe the levels. Our analyses of HDL-C levels in some serum samples supplied by the requesting laboratory confirmed the fact that their measured levels were too low. They were using a commercial kit based on the phosphotungstate-$MgCl_2$ method. The kit instructions directed the addition of 0.5 ml serum to each tube, which contained premeasured reagents. Our lipoprotein titration procedure demonstrated that the quantity of premeasured reagents present in each tube overtitrated the serum lipoproteins and caused some of the HDL to precipitate. We found that 1.0 ml was more nearly the correct volume of serum to be used with the amount of reagent in each tube. Using that volume, the requesting laboratory analyzed a set of serum samples and got HDL-C values in good agreement with our analyses of the same serum samples. This experience was reported in a leading clinical laboratory journal (17).

To assure quality control of our laboratory lipid analyses, each run included measurements of total cholesterol, HDL-C, and triglyceride levels in at least two different control samples. These controls were sometimes commercial products, but the most cost effective were serum pools prepared in-house. Small volumes of serum or plasma left after completion of routine laboratory analyses were accumulated to make pools of 500 to 1000 ml serum. Preparation, preservation, and experience using these control pools were reported at a meeting of the Aerospace Medical Association (18) to make flight surgeons aware of the economy and reliability provided by such pools.

The purpose of the quality control program was to provide prompt and unambiguous evidence if the analyses on any day's run were inaccurate or aberrant. A valuable part of the quality control program was a computer program written and implemented by this report's principal investigator. The program treated levels of total cholesterol, HDL-C, and triglycerides measured in each of the two or usually three control samples included in each day's run. The program stored the values obtained for the three lipids from the 10 most recent runs for each of the different control samples. When the current day's run was completed and entered into the computer, the printout (Table 1) listed, for each control sample: (a) the 10 most recent values for each of the three lipids; (b) the corresponding means and standard deviations; (c) the z-score (fraction of a standard deviation by which each value differed from its mean) for each value of each lipid for each run; (d) the ratios of the total cholesterol/HDL-C and total cholesterol/triglyceride for the 10 previous runs plus the current run; (e) the corresponding mean and standard deviation of each ratio; and (f) the z-score for each ratio for each run. At the conclusion of the data analysis, the stored data were updated. The oldest data were discarded and the values for the latest run were stored as the most recent values.

TABLE 1. COMPUTER PRINTOUT FOR LIPIDS QUALITY CONTROL

LIPIDS QUALITY CONTROL

Date: 12 June 1986
Control Number: 86

	Mean	Variance	Std Dev	CV
Chol	218.4	.488888	.699205	.3201492
HDLC	40.4	.2666667	.516998	1.278212
Trig	189.5	.722222	.849836	.4484626
Ratio TC/HDLC	5.40670	4.736928E-03	6.882098E-02	1.272882
Ratio TC/Trig	1.15251	1.864969E-05	4.318522E-03	.3747029

Values and Z-Scores of Last 10 Runs

Chol*	HDLC*	Trig*	TC/HDLC*	TC/Trig
218	40	189	5.45	1.159499
-.572068	-.7745996	-.5883484	.62906	.2130491
219	40	190	5.475	1.152692
.8581251	-.7745996	.5883484	.923226	2.603075E-02
219	40	191	5.475	1.146597
.858125	-.7745996	1.765045	.9923226	-1.371349
218	41	189	5.317074	1.153439
-.572068	1.161892	-.5883484	-1.302322	.2130491
219	41	189	5.341464	1.15873
.858125	1.161892	-.5883484	-.9480208	1.438233
218	41	190	5.317074	1.147368
-.572068	1.161892	.5883484	-1.302422	-1.192694
217	40	188	5.425	1.154255
-2.00226	-.7745996	-1.765045	.2658042	.4020274
218	40	190	5.45	1.147368
-.572068	-.7745996	.5883484	.62906	-1.192694
219	40	189	5.475	1.158739
.8581251	-.7745996	-.5883484	.9923226	1.438233
219	41	190	5.341464	1.152632
.8581251	1.161892	.5883484	-.9480208	2.603075E-02

Today's Values and Z-Scores

219	40	188	5.475	1.164894
.8581251	-.7745996	-1.765045	.9923226	2.865453

*Chol = Total Cholesterol = TC; HDLC = HDL Cholesterol
Trig = Triglyceride

The purpose of tabulating the 10 most recent values, associated z-scores, means, and standard deviations was to reveal, at a glance, whether the values were changing over time and whether the latest values were in accept-

able agreement with previous values. If the z-scores showed any wide excursions, these were examined to see whether the wide deviations from the mean were isolated or were part of a pattern of consistent change. Deterioration of the light filters tended to produce slowly changing trends in values and were reflected in changed instrument settings required to obtain usual values with control samples. Sloppy technician performance or instrument malfunction, such as wear or loose components in the automated pipetting mechanism; or dirt or condensate in the light path, would cause erratic fluctuation in all values and ratios. The cause of any deviations from normal values could often be inferred from the daily quality control printout. This fact made the quality control program highly useful for the purpose of quality control management. The program was discussed in a paper presented during a meeting of the Society of Armed Forces Medical Laboratory Scientists (19).

The ratios helped in pinpointing the cause of observed variations. For example, if the control sample used to set the calibration of the instrument was inaccurately diluted, then the instrument would read out correspondingly incorrect values for all the analyses. Values and z-scores of all control samples would be similarly affected (either high or low), but the ratios would be unchanged. In contrast, if the cholesterol reagent were improperly prepared, then both the total and HDL-cholesterol would be similarly affected but their ratio would be unchanged, but the total cholesterol/triglyceride ratio would be changed. Conversely, if the triglyceride reagent were faulty, the total and HDL-cholesterol levels and their ratio would be unchanged while the total cholesterol/triglyceride ratio would be changed. These patterns of relationships would be observed in all three control samples. Since such patterns would very rarely be seen because of chance variation, the ratios were powerful tools for identifying the cause of inaccuracies.

The most striking benefit from the use of this computer program was the impact on the laboratory technician running the analyses. Quality control ceased to be a threat to this individual because he/she ran the computer program. Capable technicians used the program to pinpoint the source of inaccuracies in their analyses. Subsequent runs were used to show improved accuracy and smaller variations about the mean. Bench analysts could no longer be blamed for careless dilution of control samples prepared by other personnel. For the careful technician, the computer printout was a daily documentation of the excellent quality of his/her work. Each day's run became what it should be--a source of pride in having done consistently good work. Morale soared among laboratory personnel. A report of this program and its impact was printed in a technical journal (20) and reviewed in Clinical Chemistry News (Jan 1986, p 6).

Summary of HDL-C Research

The research on HDL-C methodology provided technical expertise that generated data showing the association of CAD with elevated total cholesterol/HDL-C ratios in USAFSAM patients evaluated by coronary angiography. This expertise also generated the cholesterol and HDL-C data used in developing the USAFSAM formula for risk of CAD. Quality control for these analyses was aided by a novel computer program using lipid levels and ratios of levels in control samples to help pinpoint causes of inaccuracies in the analyses.

EFFORTS TO DEVELOP A METHOD FOR ROUTINE ANALYSIS OF PROTEIN AND LIPID CONTENT OF DISCRETE LIPOPROTEIN FRACTIONS

Results from the West Point Cardiovascular Disease Follow-up Study showed that the composition of the lipoproteins, i.e., the milligrams cholesterol per milligram protein, is significantly associated with occurrence of CAD (21). This correlation fits well with the findings of Sniderman et al. (22), who reported that some patients with CAD have a cholesterol-poor LDL in their serum. Efforts were initiated to isolate the lipoproteins of serum free from other globulins and albumin, so the amount of cholesterol per unit of apolipoprotein could be measured. While lipoproteins have been traditionally separated by ultracentrifugation in media of increased density, these lipoprotein fractions contain varying amounts of albumin and globulins. When further separation is made with the analytical ultracentrifuge, or if only lipid content is measured, these extraneous proteins do not interfere. However, if the actual amount of apolipoprotein is to be measured, a small amount of these interfering proteins can overwhelm the small protein content of the apolipoproteins and produce grossly incorrect values (Table 2, Part A). A variety of approaches made to the problem of quantitatively removing the unwanted proteins is discussed next.

TABLE 2. RECOVERIES OF CHOLESTEROL AND PROTEIN FROM HUMAN SERUM SAMPLES FRACTIONATED BY DENSITY GRADIENT CENTRIFUGATION FOLLOWED BY CHROMATOGRAPHY OVER A COLUMN OF AGAROSE GEL-ALBUMIN ANTIBODY

Part A. Recoveries from Density Gradient Centrifugation.

Fraction	Serum A % Recovery Chol	Serum A % Recovery Prot	Serum A Ratio Prot/Chol	Serum B % Recovery Chol	Serum B % Recovery Prot	Serum B Ratio Prot/Chol	Serum C % Recovery Chol	Serum C % Recovery Prot	Serum C Ratio Prot/Chol
Top	55	1.8	0.92	49	1.2	1.0	50	1.8	1.0
2	15	0.8	1.5	14	0.6	2.0	13	0.8	1.7
3	7	8	32	-	-	-	6	2	11
4	4	2	16	6	6	40	4	2	11
5	4	2	15	4	3	36	4	3	18
6	5	3	19	5	5	38	5	4	25
7	5	10	55	5	8	64	5	10	59
8	4	21	139	4	16	165	4	22	150
Bot	3	60	657	3	54	727	3	63	563
Total	107	106	31	90	94	44	94	109	33

Part A. A 0.5 ml aliquot of each of three different human serum samples was fractionated by density gradient ultracentrifugation at a speed of 30,000 rpm in a Beckman 30.2 rotor for 15 h at 15 °C. The density gradient was prepared by successively layering a centrifuge tube with each of the following solutions: 3.0 ml KBr-NaCl solution, density = 1.20; 3.5 ml NaCl solution, density = 1.063; 0.5 ml serum; and 2.0 ml distilled water. After centrifugation, fractions were harvested by removing two successive 1.0 ml aliquots from the top of the tube, then puncturing the bottom of the tube with a needle and

withdrawing the remaining fractions as successive 1.0 ml aliquots. Cholesterol levels were measured using a commercial cholesterol enzymatic reagent with an ABA 100 semiautomatic analyzer. Protein levels were measured by the Lowry (23) method. Lipoprotein content was qualitatively evaluated by agarose gel lipoprotein electrophoresis (AGLE). Cholesterol and protein recoveries are expressed as the percent of the total tube content recovered in each density fraction; i.e., (quantity recovered in the fraction/total quantity in 0.5 ml serum) x 100. The protein/cholesterol ratio is the ratio of the quantity protein recovered in fraction/quantity cholesterol recovered in fraction.

TABLE 2. RECOVERIES OF CHOLESTEROL AND PROTEIN FROM HUMAN SERUM SAMPLES FRACTIONATED BY DENSITY GRADIENT CENTRIFUGATION FOLLOWED BY CHROMATOGRAPHY OVER A COLUMN OF AGAROSE GEL-ALBUMEN ANTIBODY (CONTINUED)

Part B. Recoveries from Agarose Gel-Albumin Antibody Column.

Fraction	Serum A			Serum B			Serum C		
	% Recovery		Ratio	% Recovery		Ratio	% Recovery		Ratio
	Chol	Prot	Prot/Chol	Chol	Prot	Prot/Chol	Chol	Prot	Prot/Chol
Top	55	54	0.90	61	52	0.86	54	47	0.91
2	5	5	1.28	56	37	1.36	38	27	1.21
3	55	28	16	-	-	-	27	13	5.3
4	40	25	8	67	25	15	31	21	7
5	31	18	9	15	13	31	44	20	8
6	40	22	11	47	31	25	10	9	22
7	46	22	26	83	19	15	46	12	15
8	55	14	34	69	18	44	-	-	-
Bot	-	-	-	122	23	134	92	12	76
Total	41	20	3.0	59	26	3.1	46	16	2.3

Part B. Each density fraction collected in Part A was diluted with water and ultrafiltered in a Centricon-30 centrifuge filter tube that retained proteins but passed salts out into the filtrate. The retained proteins were diluted to a volume of 110 µl with water. A 100 µl aliquot of this solution was applied to an agarose gel-albumin antibody column. Lipoproteins were eluted in 2.0 ml 0.1 KH_2PO_4 buffer, pH 6.6. This eluate was analyzed for its cholesterol, protein, and lipoprotein content as described in Part A. Percent recoveries of cholesterol and protein are the quantity of cholesterol or protein recovered from the column/quantity of cholesterol or protein applied to the column x 100. The protein/cholesterol ratio is the ratio of the quantity protein recovered/quantity cholesterol recovered.

Agarose Gel Lipoprotein Electrophoresis

The separation of lipoproteins by agarose gel electrophoresis (24) was adapted for routine in-house (25) use because it requires only a small sample of serum, separates fractions well, and provides results in a compact form that is convenient for evaluation by densitometry or for permanent storage. Unfortunately, the best procedure includes albumin in the agarose gel preparation (24). Since removal of albumin was the aim of this research, albumin was omitted from the agarose gel preparation. The result was lower quality of electrophoresis--poorer separation of lipoprotein bands and greater tendency toward streaking of bands. Nevertheless, the albumin-less procedure was used extensively in a variety of attempts to separate pure lipoprotein fractions and to quantify their cholesterol and apolipoprotein content.

Scraping off Separated Bands for Measurement of Protein and Cholesterol Content

Duplicate slides were required for this procedure. The first was stained for lipids to identify band locations. Band positions were marked on the second slide and these areas were scraped off into separate tubes. The lipoproteins were eluted from the gel and the cholesterol and protein content was measured. Results were highly variable because of incomplete recoveries during scraping, incomplete elution from the gel, and inaccuracy in measuring the very low amounts of cholesterol eluted. This approach was not a feasible solution to the problem.

Quantitation of Protein and Cholesterol by Staining of Bands

Protein and cholesterol content of separated lipoprotein bands were evaluated by differential staining without removal from the slide. Duplicate slides were electrophoresed. One was then stained for protein, the other for cholesterol. Unfortunately, large variations occurred in measured values, particularly in levels of cholesterol. The staining of bands for cholesterol produced faint colors and unsatisfactorily low values during densitometry. Furthermore, the previously reported problems with lack of equal staining from band to band and from day to day were also encountered (26). This approach, also, was found unsuitable for routine laboratory use.

Size Exclusion Column Chromatography

Molecular weights of the lipoproteins vary from more than a million for VLDL down to less than 200,000 for HDL, with corresponding particle sizes from 80 nm down to 7 nm (27). Albumin has a molecular weight of less than 70,000 and a correspondingly small particle size. Size exclusion chromatography therefore seemed a promising approach to the separation of lipoproteins from albumin. Unfortunately, fractions eluted from columns prepared from two such media were shown by lipoprotein electrophoresis to contain albumin, particularly in the fractions that included the HDL. Despite these problems, this research approach probably would succeed if our supply system could obtain samples of the various new column media without disabling delays.

Affinity Column Chromatography

The technique of dye-ligand binding offered a likely means of removal of albumin from lipoproteins. The dye Cibacron Blue F3-GA binds to albumin. This dye, coupled to agarose gel, effectively separated albumin from other serum proteins (28). Our studies, carried out with a column of Affi-gel Blue (a commercial product of the dye bound to agarose beads), confirmed the removal of albumin, but we found that the lipoproteins were also bound to the column. Although albumin was removable by the use of a solution of NaSCN to strip the column, less than half of the cholesterol (and, presumably, the lipoproteins) was recovered. After several experiments, using different salt solutions as eluents, failed to elute the cholesterol, this technique was abandoned.

An Alternative Form of Affinity Chromatography

An alternative form of affinity chromatography was offered by the commercial availability of antibodies to lipoproteins and of column chromatography supports chemically modified to bind proteins covalently to the support material. If the antibodies on the stationary column support were chosen to bind the lipoproteins, then the interfering proteins should pass through the columns without being bound. Subsequently, the lipoproteins would have to be released from the column so that their cholesterol and protein content could be determined.

Weaknesses of this approach include the possibilities of incomplete elution of lipoproteins from the column, or contamination of eluted lipoproteins by column proteins, or the possibility that the binding of lipoproteins to the column might disturb the binding of lipids by the lipoproteins and produce aberrant lipid/protein ratios during subsequent measurements. Another problem is that if the end use of the procedure were analysis of lipoproteins in CAD patients, then antibodies to all the known lipoproteins would have to be bound to the chromatography support material in order to avoid missing some of the lipoproteins. However, the presence of an uncommon lipoprotein variant might escape detection because the chromatography support would lack an antibody to this variant. Consequently, the diagnostic procedure would fail on the patient with the unusual diagnostic problem.

Conversely, if the antibodies on the stationary phase of the column were chosen to bind albumin and unwanted globulins, then the lipoproteins should pass through the column and be available for analysis. This then was the approach chosen for study.

Antibodies to human serum albumin were purchased and covalently bound to agarose gel beads used to prepare chromatography columns. Table 2 lists results obtained from three different human serum samples. Each of these samples was first separated into nine different density fractions (Table 2, Legend). Each serum density fraction was then chromatographed over a column of agarose gel-albumin antibody. The recoveries of cholesterol and protein and the protein/cholesterol ratio in each density fraction (Table 2, Part A) and in the column eluate (Table 2, Part B) from each density fraction are listed.

The protein/cholesterol ratios in Table 2, Part A vary from approximately 1 in the top fraction to more than 500 in the bottom fraction. This distribution results from the fact that the cholesterol-rich (protein-poor) lipoproteins (VLDL and LDL) float near the top of the tube while the cholesterol-poor (protein-rich) lipoproteins (HDL) sink into the more dense solution near the bottom of the tube, where the albumin and other proteins concentrate. The protein/cholesterol ratios of purified LDL should vary between 0.6 and 0.9, while the ratios for VLDL should be even lower. For HDL, the ratios should vary between 1 and 1.8. The ratios observed in the top fractions approach the predicted values for LDL, but the ratios in the lower fractions are high and become grossly high in the bottom fractions. Such high ratios are undoubtedly due mainly to albumin, which occurs in serum at concentrations 10 to 20 times that of the lipoproteins. Transferrin and various globulins also contribute to high protein/cholesterol ratios. Obviously, the albumin and these globulins must be removed before accurate protein/cholesterol ratios for lipoproteins can be measured.

Table 2, Part B shows the effect of the albumin antibody on the cholesterol and protein content of the density fractions after these were chromatographed over the agarose gel-albumin antibody columns. The recoveries of both cholesterol and protein vary widely, reflecting the technical difficulties associated with manipulating small volumes and measuring extremely low levels of cholesterol with the progression of fraction numbers. As expected, the protein recoveries decrease as the fractions progress in density. This result fits with the expected increase in concentration of albumin in the bottom fractions. The albumin antibodies on the column should bind the albumin and give lower recoveries of protein from these eluates. Agarose gel lipoprotein electrophoresis (AGLE) of the eluates confirmed this prediction by demonstrating the absence or marked reduction of the albumin band in these eluates.

The fact that chromatography of the lower density fractions over the antibody column had little effect on the protein/cholesterol ratios suggests that those fractions contained little albumin. Since the ratios in those fractions were close to the ratios expected for VLDL and LDL, it was inferred that those fractions contained relatively pure lipoproteins. This inference was confirmed by AGLE. The other fractions, however, were contaminated by various globulins and some albumin.

To remove these proteins, antibodies to transferrin and human gamma globulins were obtained and covalently bound to agarose gel beads and then used in chromatography columns. When diluted serum was chromatographed over these columns, the target proteins were cleanly removed. This removal was demonstrated by AGLE. To facilitate one-step separation of unwanted proteins, columns were prepared using mixtures of agarose gel with antibodies to albumin, transferrin, and gamma globulins. When lipoprotein fractions, separated by density gradient centrifugation, were chromatographed over these mixed columns, the columns bound proteins, including large proportions of lipoproteins, but lost specificity for removing target proteins. This anomalous result must be an artifact of some unrecognized pitfall in the laboratory procedures. Identifying the pitfall was frustrated by the complex protocol for these experiments, which included initial density centrifugation, separation of 9 different density fractions from each serum, further separation of

each density fraction by antibody column chromatography, measurement of cholesterol and protein concentrations in all density fractions and their respective chromatography eluants, removal of salts from each of these by ultrafiltration, and monitoring of the lipoprotein content of each by AGLE. Each experiment required much painstaking technical effort and consumed more than 2 weeks. This slow progress contributed to the ultimate decision to terminate this research.

Separation of Lipoproteins by High-Pressure Liquid Chromatography

The possibility of using high-pressure liquid chromatography (HPLC) to separate serum lipoproteins was explored in cooperation with Dr. Harvey Schwertner (USAFSAM/NGIS). Preliminary experiments demonstrated separation of LDL and HDL, but the columns became plugged and lost their resolving power, and new columns suffered the same fate after fewer runs than expected. Since many of these runs were for standardization or parameter exploration, the number of useful runs was distressingly few for column combinations costing $1500 or more. Shifting to columns used successfully by Williams and Kushwaha (29) for the same purpose resulted in much longer column life, at less cost, and with better separation of fractions. The effluent stream from these columns was collected by lipoprotein fraction and analyzed for protein and cholesterol content. Amazingly, the results indicated no greater purification than had been achieved by hand-operated columns in the early phases of this research. At this point, an administrative decision was made to end this research. The problem of how to prepare lipoprotein fractions free from albumin and other serum proteins, therefore, remains unsolved.

RECOMMENDATIONS FOR FUTURE RESEARCH

Current worldwide research in the field of lipoproteins has uncovered a bewildering array of genetic variations. Uncertainty about the clinical import of these variants has led to mass confusion on how to interpret these results in predicting risk of CAD. Some sort of ordering system that will incorporate the minutiae into a meaningful summary is essential. A prime candidate for such a summary is a measurement of the serum level of the major lipoprotein classes and computation of the cholesterol load per unit of apolipoprotein. The West Point Study data clearly show that such an index is strongly predictive of risk of CAD in that study group.

To pursue the goal of separating purified apolipoproteins by density classes, it is recommended that:

1. Lipoproteins first be separated from the bulk of albumin and unwanted proteins by ultracentrifugation or electrophoresis;

2. Separated fractions be further separated using antibodies in a procedure compatible with measurement of both protein and lipid levels in each separated fraction;

3. The results be combined in a summarizing lipid transport profile or score to be meaningfully related to risk of CAD, perhaps by correlation with the degree of narrowing of coronary arteries observed in patients undergoing

coronary angiography. The lipid transport profile of patients with positive findings during coronary angiography could be compared with the profile of patients with negative findings.

ACKNOWLEDGMENTS

Coworkers and contributors to this work unit include N.J. Garcia, J.R. Hickman, Jr., H.A. Maldonado, J.W. Pierce, W.A. Rankin, P.R. Rozell, R.G. Troxler, G.S. Wiley, and J.A. Wood. In addition, the skillful laboratory work of Emmanuel L. Mosser and Fred H. Wilson (deceased) is particularly acknowledged.

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