A COMPARISON OF METHODS FOR THE

DETERMINATION OF LOW DENSITY

LIPOPROTEIN CHOLESTEROL

By

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CHAPTER I

INTRODUCTION

Coronary heart disease (CHD) is a major public health problem in the United States. Despite substantial success in reducing premature deaths from CHD in the past two decades, this disease continues to kill more than 500,000 Americans annually and is still our nation's leading cause of death and disability. About one million Americans suffer myocardial infarctions each year, and more than six million have symptoms of CHD (43). In addition, significant degrees of asymptomatic CHD are very common in our population. The impact of the illness on the economy has been estimated to be over $50 billion annually for care and lost earnings and productivity related to CHD (42).

CHD is the result of atherosclerosis, in which deposits of cholesterol and other lipids, together with cellular reactions, thicken artery walls. This process gradually reduces the lumen of the artery and restricts blood flow. Inadequate blood flow can cause injury to or death of tissue beyond the site of reduced flow. On the coronary arteries, this leads to myocardial infarction or sudden death. Many factors influence not only whether a person will develop CHD but also how rapidly atherosclerosis progresses. Genetic predisposition, gender, and advancing age are recognized as

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major risk factors for CHD that cannot be modified. High
blood cholesterol, cigarette smoking, and high blood
pressure are considered the primary risk factors for CHD
which can be modified through lifestyle changes (42).

Cholesterol is a fat-like substance (lipid) that is a
key component of cell membranes and a precursor of bile
acids and steroid hormones. Typical of lipids, cholesterol
and triglycerides are not water soluble. In order to be
solubilized in the blood and transported through the body
the cholesterol and triglycerides are bonded to protein
macromolecules. The combinations are called lipoproteins.
There are three major examples in the blood, namely HDL-C or
high density lipoprotein cholesterol, LDL-C or low density
lipoprotein cholesterol, and VLDL-C or very low density
lipoprotein cholesterol. Total serum cholesterol found in
the normal fasting individual is distributed as follows:
HDL-C 20 to 30%, LDL-C 60 to 70%, and VLDL-C, which are
largely composed of triglyceride, 10 to 15% (41). The serum
cholesterol level is determined partly by inheritance and
partly by the fat and cholesterol content of the diet.
Other factors such as obesity and physical inactivity can
also play a significant role (41).

The role of the HDL-C component is to act as a type of
shuttle as it takes up cholesterol from the blood and body
cells and transfers it to the liver, where it is used to
form bile acids. The bile acids are involved in the
digestion process, with some of them passing out with the

stool, thus providing the body with a major route for excretion of cholesterol (45).

LDL-C, on the other hand, transports cholesterol from the liver to various body cells, where it is deposited for cell functions. LDL-C is very high in cholesterol, so when LDL-C levels are excessively high, it contributes to the buildup of atherosclerosis (45).

Because the risk of CHD appears to be directly proportional to the blood levels of total cholesterol and LDL-C and inversely proportional to the level of HDL-C, efforts to measure the levels of the individual blood lipids have become extremely important in recent years (15). Common clinical uses of cholesterol measurement include advising patients with regard to their risk of developing CHD based on their lipoprotein profile; monitoring a therapeutic response to exercise, weight loss, or pharmacologic intervention; and serving as a psychological reinforcement following CHD risk factor modification. An accurate and reproducible method of cholesterol determination is required for all three applications (51).

A concerted national effort to identify and treat every American adult at high risk for CHD due to high blood cholesterol levels is expected to contribute to lower CHD morbidity and mortality rates. According to the National Cholesterol Education Panel (NCEP), all adults should know their blood cholesterol level, be aware of the implications

of elevated cholesterol, and seek the help of a physician should treatment be necessary (28).

The results of the Coronary Primary Prevention Trial, the Coronary Angiography Lipid Lowering Trial of the National Heart, Lung, and Blood Institute (NHLBI), and other plasma lipid-altering trials have focused attention on the central role of lipoproteins and cholesterol in the atherosclerotic process. A review of studies using arteriography to assess the extent of stenosis highlights the important atherogenic role of LDL-C and the antiatherogenic role of HDL-C (51). The NCEP concludes that LDL-C figures offer more precise information than total cholesterol as a risk factor, and is therefore preferred for clinical decisions about interventions to lower blood cholesterol, especially in patients who may be candidates for cholesterol-lowering drugs (41). The Adult Treatment Panel of the NCEP has predicted that the LDL-C value will be the key determinant upon which a clinical decision will be based to intervene with cholesterol-lowering therapy (28).

The basis for the experimental determination of cholesterol and its fractions in any body fluid, e.g. serum, plasma, or cerebrospinal fluid, is the intensity of a color that occurs in the product(s) of a chemical reaction. At the present time, no clinical laboratories are able to measure the LDL-C fraction directly because no reaction has been found where the color produced is exclusive to LDL-C. Instead it is calculated based on measurements of total

cholesterol (TC), HDL-C, and triglycerides (TG) (45). Based on considerable evidence the VLDL-C fraction is taken to be equal to one-fifth of the TG value in which case the LDL-C concentration, the only unknown, can be estimated using the Friedewald formula (17):

$$LDL\text{-}C = TC - (HDL\text{-}C + TG/5)$$

Concentrations are usually expressed in the units milligrams per deciliter (45), although molarity (moles/L) is becoming more prevalent.

In practice, TC is measured first. Subsequently HDL-C is measured in a second test after the other lipoproteins have been removed from the sample. The removal is generally accomplished by selective precipitation with one of the commonly used reagents, such as manganese heparin, dextran sulfate, or magnesium phosphotungstate (51). There are no known precipitating reagents that will selectively remove HDL-C and VLDL-C and allow one to measure LDL-C directly. TG is measured in a third unrelated test.

If one accepts the NCEP conclusion that LDL-C is a better predictor of CHD, then there is a need for a method that will accurately and precisely determine LDL-C cholesterol levels to assess a person's risk for CHD and to monitor treatment (44).

This investigation is intended to compare the results from a new method in which LDL-C is measured directly with the well-accepted method of calculating LDL-C levels just described. The new method, referred to as the Chugaev

reaction, and the current, well-accepted method, referred to as the Allain-Trinder method, are described more fully in Chapter III. If the results of the investigation support the hypothesis that the direct measurement of LDL-C is superior to the calculation of LDL-C, it will provide a means for accurately assessing risk for CHD, and/or monitoring treatment for hyperlipidemia.

Statement of the Problem

The Allain-Trinder method does not measure serum levels of LDL-bound cholesterol, but rather calculates it based on measurements of total cholesterol, HDL-C, and triglycerides. The Chugaev reaction, in which the LDL-C levels are measured directly will be tested, and the results from both methods will be compared.

Hypothesis

There will be no significant difference in the LDL-C values obtained by the Chugaev method of direct measurement of serum and the Allain-Trinder method of calculating LDL-C.

Limitations of the Study

In order to measure LDL-C directly, a new color reaction is required. The limitations to the new process are: no attempts will be made to separate the fractions; the intensity and stability of the color are dependent on the experimental conditions; the range of cholesterol levels

that can be measured is uncertain. The most serious limitation to the study is the magnitude of the experimental errors associated with both methods.

Delimitations of the Study

1. Subjects will be volunteers who request a lipid profile/analysis from the Oklahoma State University Wellness Center. No attempt will be made to select only those volunteers at high risk for CHD.

2. Only one trial from both methods will be made for each sample.

Assumptions

1. The subjects will have fasted for at least 12 hours prior to blood samples being taken.

2. The Allain-Trinder method of determining cholesterol and its fractions is standardized according to the College of American Pathologists (CAP).

3. Reagents for the Chugaev method remain stable over time.

4. The reference materials are pure.

5. The new color reaction has no interferences from other constituents in the serum.

Definition of Terms

Conceptual

Angina pectoris. Pain in the chest and arms or jaw due to a lack of oxygen to the heart muscle, usually when the demand for oxygen is increased during exercise and at times of stress (3).

Angiography. A procedure that enables blood vessels to be seen on film after the vessels have been filled with a contrast medium (a substance that is opaque to X rays) (3).

Arteriography. Another name for angiography (3).

Arteriosclerosis. A group of disorders that causes thickening and loss of elasticity of artery walls. Atherosclerosis is the most common type (3). Commonly called hardening of the arteries (45).

Atherosclerosis. A very common form of arteriosclerosis, in which the arteries are narrowed by deposits of cholesterol and other material in the inner walls of the artery (45). It is the type of arteriosclerosis most influenced by lifestyle factors (62).

Cardiovascular Disease (CVD). All diseases affecting the cardiovascular system including coronary heart disease, atherosclerosis, high blood pressure, stroke, rheumatic fever, and rheumatic heart disease (53). Atherosclerosis is the most prevalent form of CVD (62).

Cholesterol. A steroid alcohol found in animal fats. This pearly, fatlike substance is implicated in the narrowing of the arteries in atherosclerosis (45).

Chylomicrons. The lipoprotein formed in the intestinal wall cells following digesting and absorption of fat (62). They serve primarily to transport exogenous triglycerides to tissue sites for storage and utilization (37).

Coronary heart disease (CHD). Atherosclerosis in the arteries feeding the heart muscle (62).

High density lipoprotein cholesterol (HDL-C). Cholesterol is carried by the high density lipoprotein to the liver. The liver then uses the cholesterol to form bile acids which are finally excreted in the stool (45).

Lipids. A general term used for several different compounds which include both solid fats and liquid oils. There are three major classes of lipids: triglycerides (the principal form of fat in body fat), phospholipids (important constituents of cell membranes), and sterols such as cholesterol (45).

Lipoprotein. The carrier protein for lipids (53). There are four types of lipoproteins: chylomicrons, low density lipoprotein (LDL), very low density lipoprotein (VLDL), and high density lipoprotein (HDL) (45).

Low density lipoprotein cholesterol (LDL-C). Transports cholesterol from the liver to other body cells. LDL-C is often referred to as "bad" cholesterol because it may be taken up by muscle cells in arteries and it has been

implicated in the development of atherosclerosis (45). This type of lipoprotein is derived from VLDL-C as cells remove triglycerides from them (62).

Myocardial infarction. A common form of heart attack, in which the blockage of a coronary artery causes the death of a part of the heart muscle (45).

Very low density lipoprotein cholesterol (VLDL-C). Transports triglycerides to body tissues (45). This type of lipoprotein is made by liver cells and, to some extent, by intestinal cells (62).

Functional

Fasting. A state in which a subject of the study had taken in nothing by mouth (except water) for at least 12 hours prior to a blood sample being taken.

Reference Materials. Lipoprotein fractions that were (a) separated by ultrafiltration and commercially available from Sigma Chemical Company and (b) separated by ultracentrifugation and made available by the lipoprotein laboratory of the Oklahoma Medical Research Foundation.

Description of Instruments

Centrifuge. A high-speed clinical micro-centrifuge manufactured by Allied Fisher Scientific, Model No. 56A operated at a speed of 11,500 revolutions per minute.

<u>Incubator</u>. A water-bath with close temperature control, manufactured by Precision, Model 181, and operated at an incubation temperature of 67° Centigrade.

<u>Pipettes</u>. Automatic, adjustable micro-pipettes manufactured by Rainin Instruments Co., Inc., capable of delivering sample aliquots from 10 to 1000 microliters.

<u>Spectrophotometer</u>. This instrument, a Hitachi 100-80A, measures the intensity of light transmitted or absorbed by a specimen as a function of wavelength of the incident light.

CHAPTER II

A SELECTED REVIEW OF LITERATURE

The text of Chapter I was a description of the CHD risk
factors based upon measured values of total serum
cholesterol and/or the individual lipoprotein fractions. In
this chapter the history behind the measurements and how the
conclusions were reached are described.

Review of Recent Studies

That a relationship exists between elevated blood
cholesterol and CHD has been known for nearly a century (50)
and its origin has been the focus of laboratory
investigations for over 50 years (23). With the added
ability over the last 30 years, and especially the last 15
years, to separate and investigate the various lipids and
lipoproteins in greater detail, a wider array of potential
parameters needs to be considered in reviewing the causes of
the atherosclerotic process (50).

One of the most productive of all epidemiologic
investigations was the Framingham Heart Study (FHS). The
results from this study have played a major role in
explaining the nature of CHD risk factors and their relative
importance (52). Other major study programs in North

America which include the Lipid Research Clinics Prevalance
Mortality Follow-up Study (LRCF), the Lipid Research Clinics
Coronary Primary Prevention Trial (LRC-CPPT), and the
Multiple Risk Factor Intervention Trial (MRFIT) have made
important contributions to our understanding of these risk
factors (3). These studies produced an abundance of
epidemiologic information that seem to confirm that specific
factors are directly associated with an increased risk for
the development of CHD (52).

The FHS was a prospective epidemiological study of
cardiovascular disease (CVD). Beginning in 1949 a group of
5,209 men and women, then aged 30 to 59 years, were enlisted
into a longitudinal study (21). Since then, the progress
of each volunteer has been followed by means of routine
biennial medical examinations where possible, and
and from morbidity and mortality data provided by hospitals
and other sources. The measurements of fasting HDL-C and
triglyceride levels were introduced in 1969 (21,8).

In a report of the FHS written by Gordon et al. (21)
and based upon four years of surveillance, the major potent
lipid risk factor for CHD was thought to be HDL-C, which
showed an inverse relationship with the incidence of CHD (p
< 0.001) in both men and women. An association with the
incidence of CHD (p < 0.05), but one of much less importance
was observed for LDL-C. That correlation was direct, i.e.,
not inverse.

Again, based on the FHS data, Lavie et al. (29) reported that CHD is most prevalent when LDL-C is high and HDL-C is low; it is very rare when LDL-C is low and HDL-C is high. High and low in this sense are undefined relative numbers. It was also explained, however, that even when LDL-C levels are very high, CHD is fairly uncommon if HDL-C levels are 65 mg/dL or more, and it is rare when HDL-C levels are as high as 85 mg/dL. And, on the other hand, even when LDL-C is very low (100 mg/dL), CHD is still common when HDL-C levels are also very low (25 mg/dL).

Using data from the Framingham investigation, Castelli et al. developed a relative risk score that was based on the ratio of total cholesterol to HDL-C (TC/HDL-C) (51). This risk ratio is still commonly used in clinical practice (51). A suggestion was made that the ratio of TC to HDL-C is the best predictor of developing CHD (15). To be considered at low risk, this ratio, TC/HDL-C, should be less than 5.0 in males and less than 4.5 in females. The problem with any ratio, however, is that it gives no indication by itself of the absolute values. Do ratios of 5 that are equal to 350:70 and 200:40 signify equivalent risk factors? It was proposed, therefore, that if the TC/HDL-C ratio is used for risk assessment, then absolute values must also be indicated (15).

The Lipid Research Clinics Prevalence Study was also an epidemiological study of lipid and other cardiovascular risk factors and was done during 1972-1976 in 10 collaborating

North American Centers (19). Fasting plasma lipid levels and selected medical and sociodemographic data were obtained for more than 70,000 men and women. The selected populations were deliberately diverse, covering a broad range of geographic, socioeconomic, occupational, age, sex, and ethnic groups. In 1977, a mortality follow-up study (LRCF) was begun involving all participants in the Prevalence Study who were at least 30 years old at that time (23). The primary objective of this study was to acquire data on the prevalence of difference types of hyperlipoproteinemia in various age and ethnic groups (33).

The LRC-CPPT study was a multicenter, randomized, double-blind trial of the efficacy of lowering LDL-C levels in reducing CHD risk in 3,806 asymptomatic middle-aged men with primary hypercholesterolemia (plasma cholesterol $\geq$ 265 mg/dL (31,32).

Part I of the LRC-CPPT (31) was designed to test the hypothesis that lowering total cholesterol and LDL-C by diet or drugs or both will reduce the subsequent incidence of CHD. In part II of the LRC-CPPT (32) the quantitative impact of cholesterol lowering on CHD incidence was evaluated. The combined LRC-CPPT findings confirmed that reducing total cholesterol by lowering LDL-C levels can diminish the incidence of CHD morbidity and mortality in men whose high risk for CHD is a consequence of elevated LDL-C levels. A decrement of 22.3 mg/dL in LDL-C levels was associated with a 16% to 19% reduction in CHD risk.

The MRFIT study was a randomized multicenter clinical trial to test the effect of a multifactor intervention program on mortality from CHD in 12,866 high-risk men aged 35 to 57 years. The subjects were without clinical CHD manifestations but were at high CHD risk (upper 10-15%) because of a combination of hypertension, cigarette smoking, and elevated plasma cholesterol (40).

An analysis of the MRFIT data by Stamler et al.(48) demonstrated that the relationship between serum cholesterol and CHD is not a threshold one, but a continuously graded one that is a dominant factor in assessing risk for the great majority of middle-aged American men. In other words, the conclusion is that the great majority of adults in the United States are at increased CHD risk because of their status in regard to this factor, and not only those relative few in the highest or the two highest quintiles of the distribution. Specifically, serum choleterol levels of about 180 mg/dL and above are associated with increased risk for middle-aged American men, and not just levels that are equal to or greater than 220 to 240 mg/dL.

In 1989, Gordon et al. (19) analyzed pooled data from these four large prospective epidemiologic studies (FHS, LRCF, LRC-CPPT, and MRFIT), and a conclusion was reached that for every 1 mg/dL rise in HDL-C, the CHD risk dropped about 2% in men and 3% in women, and cardiovascular mortality decreased by 4% in men and 5% in women.

Lavie et al. (29) seemed to agree with Gordon and others that HDL-C is the most important lipid risk factor in assessing the risk for CHD, even more important than TC or LDL-C. A strong case is presented for emphasizing the measurement of HDL-C in adults and for treating most patients with low HDL-C levels.

Establishment of the National
Cholesterol Education Program

A large body of evidence of many kinds has linked elevated blood cholesterol levels to CHD (10). However, many doubts still remain about the weight of the evidence for a cause and effect relationship. To resolve some of these questions, the National Heart, Lung, and Blood Institute (NHLBI) and the National Institutes of Health (NIH) Office of Medical Applications of Research convened a Consensus Development Conference on Lowering Blood Cholesterol to Prevent Heart Disease in 1984.

Based upon a series of expert presentations and reviews of all of the available data, a consensus panel reached the following conclusions: the elevation of blood cholesterol levels is a major cause of coronary artery disease; and it has been established beyond a reasonable doubt that lowering elevated blood cholesterol levels (specifically, blood levels of LDL-C) will reduce the risk of heart attacks caused by CHD (10).

Among the recommendations arising from this conference were: individuals with high-risk cholesterol levels must be identified and treated; changes in eating patterns for members of the general public must be developed and encouraged; and a national cholesterol educational program must be created and implemented. In response to this the National Cholesterol Education Program (NCEP), which the NHBLI had begun to plan in early 1984 was launched in November 1985 (42). The goal of the NCEP was to reduce the prevalence of elevated blood cholesterol in the United States, and thereby contribute to the reduction of CHD morbidity and mortality.

Since its inception the NCEP has issued periodic reports developed by its Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel or ATP) and its Laboratory Standardization Panel (LSP) on the validity of measurements (42). In addition, the Population Panel issued a report based on an intensive review of the scientific bases for making blood cholesterol-lowering recommendations and particularly eating pattern recommendations that are offered to the general public. A fourth panel will report later on blood cholesterol in children and adolescents (42).

In 1987, the NCEP Adult Treatment Panel recommended that all U.S. citizens older than 20 years have their cholesterol level tested. A desirable total cholesterol level was defined to be below 200 mg/dL, borderline-high is

in the range 200-239 mg/dL, and high risk at levels above
240 mg/dL. Similarly a desirable LDL-C value was defined to
be below 130 mg/dL, borderline-high from 130 to 159 mg/dL,
and high risk at levels above 160 mg/dL. For patients with
multiple risk factors, including history of CHD or two other
known risk factors, intervention at even lower levels of
cholesterol was recommended (41).

In summary, the Adult Treatment Panel report has given
priority to the treatment of elevated LDL-C concentratons
but has not ignored the importance of low HDL-C levels. The
report designated low HDL-C level as a major risk factor for
CHD and recommended that HDL-C be measured for any patient
deemed to be at high risk for CHD. The rationale for focus
on elevated LDL-C concentrations is based on strong
scientific evidence (25). Data that indicate increased
levels of LDL-C is a major atherogenic factor are derived
from several types of epidemiologic studies, from clinical
evidence in patients with familial hypercholesterolemia,
from investigations in experimental animals, from analysis
of pathologic specimens, and from recent studies in tissue
culture (25).

The panel also determined that therapeutic reduction of
high LDL-C concentrations will decrease the risk for CHD.
Clinical trials have shown that lowering serum LDL-C levels
by diet or drugs will reduce the incidence of CHD. Because
of this combined evidence for causation and therapeutic
benefit, the Adult Treatment Panel concluded that the major

emphasis on therapy for cholesterol as a risk factor should be directed toward patients with high levels of LDL-C (25).

The NCEP's emphasis on the importance of LDL-C as a risk factor for CHD as opposed to the TC/HDL-C ratio established by the Framingham investigation is based on the following rationale. Several opinions have been expressed about the validity of HDL-C data in relation to CHD, many of which are nonsupportive.

If HDL-C is to become part of a standard risk profile for CHD, great care must be given to the precision in the laboratory measurements. A good laboratory can achieve a technical error of 5 mg/dL in measuring this lipid. But when it is remembered that an average HDL-C level for adult men is around 45 mg/dL and a significantly high risk of CHD is evident at 35 mg/dL, it is clear that a technical error of 5 mg/dL is by no means a comfortable one (21).

In addition, since HDL-C is subtracted in determining LDL-C, the errors are reciprocal, substantially compromising the overall estimation of CHD risk because the risk relationships are also opposite (56).

The accurate determination of HDL-C values requires constant attention to detail and adequate quality control. Even in a proficient laboratory, the absolute limit of reproducibility of HDL-C measurements may limit the way patient's values can be clinically used (51).

Grundy, et al. (25) cite the following reasons for not recommending universal screening for low levels of HDL-C:

(1) In the absence of CHD or other risk factors, the risk for CHD is not increased markedly in those who have TC levels in the range of 200-239 mg/dL, compared with the risk at levels below 200 mg/dL.

(2) Laboratory costs associated with generalized testing would increase. All persons tested would require at least two tests (TC and HDL-C) and probably three tests (TC, HDL-C, and TG, with calculation of LDL-C). Interpretation and discussion by the physician would also increase costs.

(3) The methods for estimating HDL-C have not been well standardized. Some current methods systematically underestimate true HDL-C levels which will result in an excessive number of individuals being classified as having low HDL-C levels. An uncertainty of a few milligrams per deciliter in HDL-C has little effect on the clinical interpretation of the estimated LDL-C level, but a relatively small error can have an important affect on the interpretation of the clinical significance of the HDL-C levels.

Warnick (56) adds that accuracy in the HDL-C measurement is of particular importance since HDL-C is a powerful inverse predictor of CHD risk which is expressed over a narrow concentration range. The NCEP recommended cutpoint of 35 mg/dL differs only little from the usual population mean of approximately 50 mg/dL.

Superko et al. (51) investigated the difficulties inherent in determining HDL-C values, and concluded that

often HDL-C measurements lack sufficient accuracy to be of practical use in an individual clinical setting.

Frolich et al. (18) report that the current problems with the accuracy and precision of the serum HDL-C assay prevent it from being the single most important test for assessment of the lipid risk factors for CHD.

Laboratory Standardization Panel
Recommendations

In 1988 the Laboratory Standardization Panel (LSP) of the NCEP defined and established goals for precision and accuracy of TC, TG, and HDL-C measurements to minimize the effect of laboratory error (28). Accuracy refers to the "closeness to the true value" while precision reflects the test-to-test and day-to-day reproducibility (18). Without these defined goals and suitable reference standards, accurate classification of risk is meaningless (7).

The LSP recommends that, as a national goal, clinical laboratories should initially achieve an overall precision consistent with a coefficient of variation (CV) of $\pm5\%$ or less; ultimately, laboratories should achieve a CV of $\pm3\%$ or less. CV is defined to be a relative measure of precision and is equal to the standard deviation of a set of values divided by the mean, which when multiplied by 100 can be expressed as a percentage (28).

The LSP recommends that biases (departures from the true value) in methods presently in use should not exceed

$\pm5\%$ and that ultimately a national goal of $\leq 3\%$ bias should be achieved (28). By this definition, bias is a quantitative measure of the degree of inaccuracy. The difference between the true, accepted, or expected value and the observed value, is expressed either in the units of the measurement or as a percentage.

Accuracy and precision are of vital importance in assessing serum cholesterol levels (18). Unfortunately, accuracies are very low and imprecisions of measurements are very high. Even the measurement of total serum cholesterol is fraught with problems and it is now apparent that the goal of $\pm3\%$ inaccuracy and precision may lead to misclassification of large numbers of patients (18). Consider the sliding scale for CHD that is based upon those levels: < 200 mg/dL, 200-239 mg/dL, and $\geq$240 mg/dL. The middle range is only 40 mg/dL wide and in order to get a 95% confidence level in the risk assignment, the accuracy and the bias must both be $<\pm3\%$ (61).

Future Goals in Measurement

LDL-C has been recommended by the NCEP Adult Treatment Panel as the determining factor in initiating dietary and drug treatment (14). Considering its importance, the methodology for the measurement of LDL-C is substantially lacking. The only convenient routine is to calculate it from known values for total cholesterol, HDL-C, and VLDL-C levels as the remainder in the Friedewald formula (56).

Laboratory performance specifications have not been established for LDL-C cholesterol (56).

Methods for quantitation of the lipoprotein risk factors are steadily improving, but work still remains to be done to achieve acceptable routine performances by diagnostic laboratories. Ultracentrifugation, the only known method available for the direct separation of LDL-C, is tedious, expensive and requires a large specimen volume (34, 56, 57, 60); validation of a simpler whole serum method with equivalent results would be desirable (56).

Based on the importance of LDL-C in risk classification and treatment, better methods for quantification of LDL-C cholesterol, especially direct methods, are needed (56).

Summary

The positive association of LDL-C and total serum cholesterol and the negative association of HDL-C with CHD risk are well established. From the literature reviewed in this chapter, it is evident that there is considerable difference of opinion as to which of these factors is the strongest predictor of coronary heart disease. The pooled data from the four major studies reviewed (Framingham Heart Study, Lipid Research Clinics Prevalance Mortality Follow-up Study, Lipid Research Clinics Coronary Primary Prevention Trial, and the Multiple Risk Factor Intervention Trial) showed a powerful inverse relationship between HDL-C and the likelihood of developing CHD. However, as pointed out, the

difficulties inherent in determining HDL-C values limit its value as an important lipid risk factor in assessing the risk for CHD.

The National Cholesterol Education Program acknowledged the importance of HDL-C levels of less than 35 mg/dL as a coronary risk factor, but stated that LDL-C offers more precision as a risk factor and is therefore preferred for clinical decisions about interventions to lower blood cholesterol. Accepting the recommendations of the NCEP that LDL-C is the better predictor of CHD, it was determined to be the focus of this study.

CHAPTER III

METHODS AND PROCEDURES

This study compared the results from a method developed to directly measure the LDL-C fraction of serum cholesterol with the results from a well-accepted method of calculating LDL-C levels. The methods and procedures for collecting samples and preserving patient anonymity were approved by the Oklahoma State University Institutional Review Board.

Analytical Detection of Cholesterol

Cholesterol levels cannot be measured without first reacting the molecule to form a colored derivative whose intensity can be measured and is known to be proportional to the amount of cholesterol present. The well-accepted method, also the only convenient method, was developed by Allain in 1974 (2) and uses a color derivatization reaction described by Trinder in 1969 (55). In all subsequent discussions the reaction will be referred to as the Allain-Trinder method.

The contribution from Allain was the two-step double enzymatic reaction using a single reagent system (2) in which cholesterol is derivatized to cholest-4-en-3-one according to:

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$$\text{Cholesterol Esters} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$
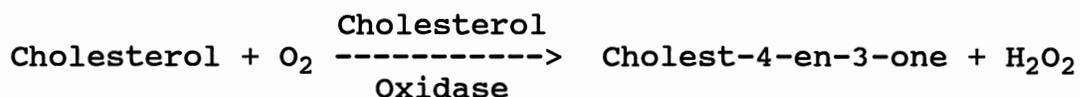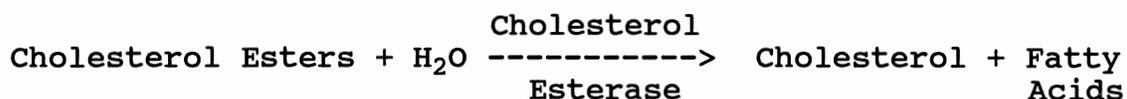
The hydrogen peroxide produced in the Allain reaction becomes a reagent in the Trinder reaction. The product of interest is the red-colored quinoneimine dye.

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzenesulfonate}$$

$$\xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$
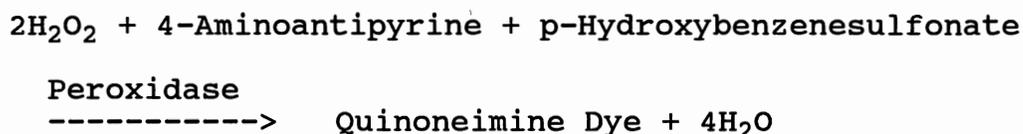
This is the current, "state-of-the-art" method for cholesterol measurement. This is also the procedure to which all of the imprecision problems previously described are related.

The quinoneimine dye has a visible absorbance maximum at 500 nm. The intensity of the color produced is directly proportional to the total cholesterol concentration in the sample because of the one-to-one relationship in the second equation between cholesterol and H_2O_2.

This reaction is done at 37° C, which is normal body temperature and the temperature at which the enzymes function. Temperature and incubation time for the reactions were chosen that produce the most color intensity and color stability in the quinoneimine dye.

Of the three lipoprotein cholesterol fractions only the HDL-C can be measured directly. The low density fractions are selectively removed from the total serum by adding a precipitating agent. Those commonly used and approved by the various regulatory agencies are manganese heparin (57), magnesium phosphotungstate (4), and dextran sulfate (58). Heparin-manganese is the precipitating agent recommended by the NCEP and Centers for Disease Control (CDC). Because it involves a protracted and complex procedure, it is used primarily in regulatory laboratories and it is not used for broad screening programs. Magnesium phosphotungstate is the most common precipitating agent used, but it is sensitive to separation conditions. Dextran sulfate is much more convenient to use in routine screening, and it is the precipitating agent used by Roche Biomedical Laboratories. This agency is the source laboratory for Allain-Trinder data in this work. There are numerous possible sources of error in the measurement of the fractions with the enzymatic method, including the following:

1. Cholesterol is not measured directly; rather, it is assumed that the number of H_2O_2 molecules produced by the reaction is equal to the number of cholesterol molecules entering the reaction. It is the H_2O_2 that reacts with the chromogen and produces the color, not the cholesterol. H_2O_2 is known to be an unstable compound and is not a selective redox agent.

2. Serum is not homogeneous, which may result in sample inconsistencies. In other words, it could oxidize other compounds that might be present in random serum samples.

3. Only one measurement was performed on each sample.

4. Precipitation of LDL-C and VLDL-C may not be complete or totally selective.

5. The estimation of VLDL-C as being TG/5 is not always accurate, particularly when triglycerides are in excess of 400 mg/dL.

Measurement of total cholesterol is also subject to error due to the following:

1. The same problem involving H_2O_2 described above.

2. Blood cells may be lysed during the reaction, which produces a red color in the serum that can interfere with the absorption measurement at 500 nm.

The proposed method, referred to as the Chugaev reaction, is an attempt to reduce these errors in measurement. The method was first described in the chemical literature in 1910 (9). The reagent is a 2:1 mixture of 27% $ZnCl_2$ in glacial acetic acid and 98% acetyl chloride. The reaction is done at elevated temperatures and the color is produced by the cholesterol molecule directly. The method is non-enzymatic and distinction among the fractions is based upon the selectivity of the reagent for cholesterol in different lipid environments.

The intensity of the color produced by the reaction is measured using absorption spectrophotometry. A source of

white light is directed through a prism which separates the light into the colors of the spectrum ranging from red (750 nm) to violet (360 nm). Individual wavelengths are selected by rotating the prism. The beam is led through a slit opening and illuminates a cuvet which contains the serum sample. The result is a representation of the absorption spectrum on paper with absorbance on the y axis as a function of the wavelength in nanometers on the x axis.

Generally speaking, the linear relationship that exists between the color intensity (absorption) and the quantity of material is determined through the use of standard references in which the exact amounts of the materials are known. Concentrations of cholesterol were calculated from the absorbance measurements made at selected wavelengths.

Hazards of the Method

Standard precautions for handling human blood samples were observed during the experiment. Since both acetyl chloride and $ZnCl_2$ are corrosive and toxic substances, additional precautions were taken: sealed containers were used at all times, and all work was done under a fume hood. The blood samples and reagents were disposed of according to Occupational Safety and Health Administration (OSHA) regulations.

Testing Procedures

The subjects were volunteers who requested a lipid
profile from the Oklahoma State University in September,
October, and November, 1991. No attempt was made to select
subjects according to demographic classification, and no
demographic data was collected. They were instructed to
report to the Wellness Center laboratory having fasted for
at least 12 hours previous to their arrival. Written
informed consent, as shown in Appendix A, was obtained from
each subject in accordance with institutional guidelines.

Clinical Laboratory Procedures

A venous blood sample was drawn from the brachial fossa
of either the right or left arm of each subject. A standard
aseptic venipuncture technique was employed with the
tourniquet being released prior to removal of the #21 gauge
needle. All subjects were in the sitting position during
venipuncture. Vacutainer^{tm} red stoppered tubes (serum
separation tubes, SST) were used in venous collection.
These have a floating gel to aid in separation of the red
cells from the serum. One tube, approximately 10 mL, per
subject was collected.

All venous samples were allowed to stand at room
temperature for a minimum of 30 minutes and a maximum of 1
hour 30 minutes until a clot formed in the tube. The
samples were then centrifuged at a speed of 5,000
revolutions per minute for ten minutes in a table top

clinical centrifuge (Roche Biomedical Laboratories VanGuard 6000). The gel separated the red cells from the serum.

A 1 mL aliquot of the serum was aspirated using a variable volume Rainin Pipetman[tm] and transferred into a new 10 mL glass vial with screw cap. This portion of the sample was taken to the Oklahoma State University Department of Chemistry, Room B002 Physical Sciences Building I, for measurement by the Chugaev method. The Vacutainer[tm] tubes were collected by Roche Biomedical Laboratories personnel for measurement according to the Allain-Trinder method described above, at its Kansas City, Missouri, regional laboratory.

The Chugaev reaction was performed on 10 microL of serum. To this a 1 mL aliquot of 98% acetyl chloride (Aldrich Chemical Co.) and a 50 microL aliquot of 27% $ZnCl_2$ in glacial acetic acid were added. The vial was capped, and on shaking the mixture a protein precipitate was formed. The vial was placed in a 67° C water bath and incubated for 8 minutes. The product of the reaction is an orange or reddish-orange colored solution. The vial was removed and cooled in a room temperature water bath. The contents were transferred to a 1.7 mL polypropylene centrifuge tube, sealed, and centrifuged at 15,000 rpm for two minutes.

The supernate was transferred to a 1 cm. pathlength cuvet and placed in the cell compartment of the Hitachi 100-80-A spectrophotometer. The visible absorption spectrum was run from 700 nm to 400 nm. The spectrum was corrected for

solution blank and instrument baseline by subtracting this
spectrum, which was saved in the computer memory of the
spectrophotometer, from the spectrum for the colored product
of the Chugaev reaction. A typical printout of the net
spectrum for whole serum cholesterol and the three fractions
is shown in Figure 1. Since the Chugaev reagent combines
with cholesterol in all of its biological environments in
human serum, the spectrum is the weighted aggregate of the
contributions from cholesterol bonded to the three major
lipoprotein fractions, namely, the VLDL-C, LDL-C, and HDL-C.
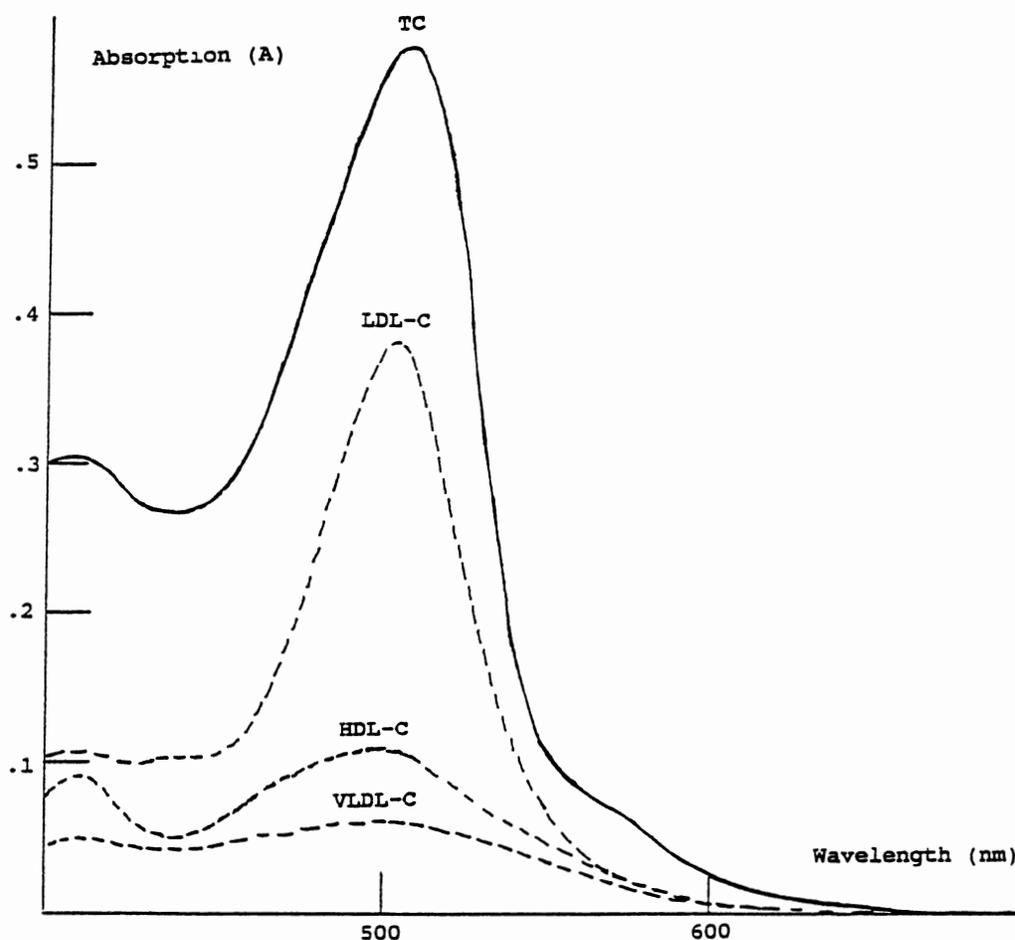
Figure 1.   Typical Printout of Spectrum for Total
            Cholesterol, LDL-C, HDL-C and VLDL-C

Analysis of the Spectrum

for the Fractions

In the Allain-Trinder enzymatic method the colors
produced for the three fractions are identical. Because of
this, separations are necessary before the distribution of
cholesterol among the fractions can be determined. The
success of the Chugaev method depends entirely upon the fact
that the colors, and therefore the absorption spectra for
the products of the reactions with cholesterol in each of
the fractions, differ. It is proposed that this will enable
the researcher to determine the total distribution in one
experiment.

In order to substantiate this hypothesis, the reaction
was run on lipoprotein fractions that were (a) separated by
ultrafiltration and commercially available from Sigma
Chemical Company and (b) separated by ultracentrifugation
and made available by the lipoprotein research laboratory of
the Oklahoma Medical Research Foundation. The absolute
purity of the fractions as standard reference materials was
not guaranteed, but the samples are among the best that are
available. Absorption spectra for each of the fractions are
in fact significantly different as shown in Figure 1, and it
is theoretically possible to use these differences to
quantitatively calculate the amounts of each fraction in a
total serum cholesterol spectrum. The remaining problem is
to find a mathematical model that will fit the spectra for

the weighted contributions from each cholesterol fraction to the total spectrum of the whole.

Mathematical Model

The first assumption made was that the contributions from each of the fractions are additive at all wavelengths from 700 nm to 400 nm. Therefore, the absorption at any wavelength is a weighted sum of three parts.

According to the theory of light absorption, also known as Beer's Law (47), the magnitude of the absorption (A) is related directly to the absorption strength (E), the concentration of the absorbing molecule in the solution (C), and to the pathlength of the solution in the cuvet (d) and is given by the simple equation

$$A = ECd$$
(Equation 1)

The quantity E depends upon the molecular structure of the absorbing compound and cannot be calculated. It is usually measured from the slope of a linear plot of A versus C at constant d (47). At every wavelength, therefore, there will be three absorption terms:

$$A = A_{VLDL-c} + A_{LDL-c} + A_{HDL-c},$$

and if these are substituted by the Beer's Law equivalents, the total absorption is given by the equation

$$A = E_{VLDL-c}C_{VLDL-c}d + E_{LDL-c}C_{LDL-c}d + E_{HDL-c}C_{HDL-c}d$$
(Equation 2)

To solve the problem, the E values must be known. However, because the fractions are not pure, accurate values

can not be determined from the linear dependencies of A versus C. Consequently, E values must be estimated empirically, and in order to determine the unknown concentrations C_{LDL-C}, C_{VLDL-C} and C_{HDL-C}, absorption measurements must be made at three different wavelengths. Overall therefore, nine E values are required to determine the distribution of cholesterol among the three fractions. The simplest mathematical model is to solve three simultaneous linear equations.

<div align="center">

Selection of Wavelengths and

Determination of E Values

</div>

In theory, the optimum wavelengths to use for quantitative work are turning points in the absorption spectrum, because errors in the measurements are minimized. These are often distinguished as maxima or minima in absorption values. The wavelengths selected for the mathematical model were the maximum at 518 nm, the minimum at 450 nm, and the maximum at 420 nm, as shown in Figure 1. The wavelengths are typical of the turning points for the spectra for all serum samples, although they do not exactly correspond with the turning points for all of the fractions individually.

For a solution of known concentration (C), and known pathlength (d), the absorption measurements are directly proportional to the E values (Equation 2). In all of this work d = 1 cm. Therefore, if an E value can be estimated at

any one wavelength, then it can be calculated at all other wavelengths in the spectrum. The assumption was made that at the major maximum at 518 nm the E values for VLDL-C, LDL-C, and HDL-C were equal. Using this value and the spectra for each of the fractions, the six E values could be calculated at the remaining two wavelengths for each fraction. Substituting the single value for E into the absorption equation, Equation 2, at 518 nm, the expression can be rewritten as:

$$A = E[C_{VLDL-c} + C_{LDL-c} + C_{HDL-c}] = E(TC).$$

Given a value for TC and a measured A value for a serum sample, E at 518 nm can be determined.

In order to calibrate the spectrum and to determine E at 518 nm, measured TC values from Roche Laboratories were used. Statistically a single value cannot be used for calibration because of the random errors associated with a single measurement. Consequently, the ratios of measured A values divided by the TC values, as determined by Roche, for the 77 subject samples were averaged. The value for E at 518 nm was determined to be 3.00 ± 0.10 mA.dL/mg. This value is used as the basis for the calculation of the other E values for all fractions using the spectrum data for each. For instance, E_{LDL-c} at 450 nm is equal to

$$\{A_{LDL-c(450)}/A_{LDL-c(518)}\}[E_{LDL-c(518)}]$$

The resultant E values in mA.dL/mg are:

FRACTIONS	VLDL-C	LDL-C	HDL-C
E_{518}	3.00	3.00	3.00
E_{450}	1.35	1.25	1.97
E_{420}	2.41	1.25	2.52

and the corresponding linear simultaneous equations required for the mathematical model are:

$$A_{518} = 3.00\ C_{VLDL-c} + 3.00\ C_{LDL-c} + 3.00\ C_{HDL-c}$$

$$A_{450} = 1.35\ C_{VLDL-c} + 1.25\ C_{LDL-c} + 1.97\ C_{HDL-c}$$

$$A_{420} = 2.41\ C_{VLDL-c} + 1.25\ C_{LDL-c} + 2.52\ C_{HDL-c}$$

The E values obtained in this way are not exact because the separations of the individual fractions by either ultracentrifugation or ultrafiltration are not exact. This is the major obstacle to accurate measurements of serum cholesterol fractions. Consequently in subsequent comparisons between results determined by the Chugaev and the enzymatic methods, additional minor adjustments in the E values might be necessary. While this may affect the accuracy of the measurement it will not affect the level of precision that can be achieved with the Chugaev method. Precision is established by the reproducibility of the spectrum for the serum samples and not by the mathematical model or the individual E values.

The A values were measured for each serum included in the study at all three wavelengths. Data were entered into a Wingz^{tm} software spreadsheet for the MacIntosh computer which includes an algorithm program to solve the three

simultaneous linear equations for the amounts of each fraction in mg/dL.

Statistical Analysis

The data collected in the study were analyzed by the following methods:

1. Pearson r between the pairs of scores for each dependent variable was calculated.

2. The percent of each fraction to total cholesterol for each subject and the average percent for each fraction was calculated.

3. The values from one method (Chugaev) were subtracted from the values of the other method (Allain-Trinder) and the differences were averaged.

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this study was to test the hypothesis that there would be no significant difference in the LDL-C values directly measured by the Chugaev method and calculated values derived from the Allain-Trinder enzymatic method.

Each of the 77 subjects requested a lipid profile/analysis from the Oklahoma State University Wellness Center during September, October, and November of 1991. They were instructed to report to the Wellness Center having fasted for at least 12 hours.

The data collected in the study were analyzed by the following methods:

1. Pearson r between the pairs of scores for each dependent variable was calculated.

2. The percent of each fraction to total cholesterol for each subject was calculated, and the average percent of each fraction to total cholesterol was calculated.

3. The values from one method (Chugaev) were subtracted from the values of the other method (Allain-Trinder) and the differences were averaged.

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Results

The raw data by subject number are given in Appendix B.
The normative data are given in Table I.

TABLE I

NORMATIVE DATA

Variable	Allain-Trinder			Chugaev		
	Mean	SD	SE	Mean	SD	SE
	(in mg/dL)			(in mg/dL)		
TC	200.5	36.5	4.2	198.2	41.3	4.7
HDL-C	46.0	12.9	1.5	45.8	14.2	1.6
VLDL-C	21.9	10.7	1.2	17.1	7.8	0.9
LDL-C	131.9	32.5	3.7	135.4	35.2	4.0

TC   = total cholesterol
HDL-C   = high density lipoprotein cholesterol
VLDL-C = very low density lipoprotein cholesterol
LDL-C   = low density lipoprotein cholesterol

Results of Pearson r Analysis

The results of the Pearson r analysis are given in
Table II. The correlations between the two methods for two
of the variables were significant ($p < .01$) while the other
two were not.

TABLE II

CORRELATION COEFFICIENTS*
n = 77

Allain-Trinder TC	vs.	Chugaev TC	.9464
Allain-Trinder HDL-C	vs.	Chugaev HDL-C	.0059
Allain-Trinder VLDL-C	vs.	Chugaev VLDL-C	-.0158
Allain-Trinder LDL-C	vs.	Chugaev LDL-C	.8555

* $r = .22$ with $p < .05$
  $r = .29$ with $p < .01$

Results of Percent Fraction Analysis

The results of the percent fraction analysis are given in Table III.

TABLE III

CHOLESTEROL FRACTION AS A PERCENT
OF TOTAL CHOLESTEROL

	Chugaev		Allain-Trinder	
	Mean (in mg/dL)	(%)	Mean (in mg/dL)	(%)
TC	200.5		198.2	
HDL-C	46.0	(23.7)	45.8	(23.7)
VLDL-C	21.9	(10.8)	17.1	(8.5)
LDL-C	131.9	(65.2)	135.4	(67.6)

TC =   total cholesterol
HDL-C =   high density lipoprotein cholesterol
VLDL-C = very low density lipoprotein cholesterol
LDL-C =   low density lipoprotein cholesterol

Results of Difference Analysis

The results of the difference analysis are given in Table IV. The frequency distributions for the difference analyses are given in Tables V, VI, VII, and VIII.

TABLE IV

MEAN DIFFERENCE BETWEEN METHODS
(ALLAIN-TRINDER MINUS CHUGAEV)

TC	2.299 mg/dL
VLDL-C	4.857 mg/dL
HDL-C	.286 mg/dL
LDL-C	-3.519 mg/dL

TABLE V

FREQUENCY DISTRIBUTION FOR DIFFERENCE
(ALLAIN-TRINDER TC MINUS CHUGAEV TC)
n = 77

Value (in mg/dL)	Frequency	Percent	
-28.00	1	1	
-25.00	1	1	
-24.00	1	1	
-18.00	2	3	
-17.00	1	1	
-14.00	2	3	
-13.00	1	1	
-12.00	1	1	
-11.00	2	3	
- 9.00	4	5	
- 8.00	3	4	
- 7.00	4	5	
- 6.00	2	3	
- 5.00	1	1	
- 3.00	6	8	
- 2.00	1	1	
- 1.00	2	3	
.00	1	1	
1.00	2	3	
2.00	1	1	Mean 2.299
3.00	4	5	SD 13.624
4.00	3	4	
6.00	1	1	
7.00	4	5	
8.00	4	5	
9.00	2	3	
10.00	2	3	
12.00	2	3	
14.00	1	1	
15.00	1	1	
16.00	2	3	
18.00	2	3	
19.00	1	1	
20.00	1	1	
21.00	2	3	
22.00	1	1	
24.00	1	1	
25.00	1	1	
31.00	1	1	
34.00	1	1	
39.00	1	1	

TABLE VI

FREQUENCY DISTRIBUTION FOR DIFFERENCE
(ALLAIN-TRINDER VLDL-C MINUS
CHUGAEV VLDL-C)
n = 77

Value (in mg/dL)	Frequency	Percent	
-20.00	1	1	
-15.00	2	3	
-13.00	1	1	
-12.00	2	3	
-11.00	2	3	
-10.00	2	3	
- 8.00	3	4	
- 7.00	1	1	
- 6.00	3	4	
- 5.00	2	3	
- 4.00	3	3	
- 3.00	4	5	
- 2.00	3	4	
- 1.00	1	1	
.00	4	5	
1.00	2	3	
2.00	1	1	
3.00	4	5	
4.00	4	5	Mean 4.857
5.00	2	3	SD 13.371
6.00	1	1	
7.00	1	1	
8.00	3	4	
9.00	2	3	
10.00	2	3	
11.00	3	4	
13.00	1	1	
14.00	1	1	
16.00	2	3	
17.00	1	1	
19.00	1	1	
21.00	2	3	
25.00	2	3	
27.00	2	3	
29.00	1	1	
31.00	2	3	
34.00	1	1	
38.00	1	1	
39.00	1	1	

TABLE VII

FREQUENCY DISTRIBUTION FOR DIFFERENCE
(ALLAIN-TRINDER HDL-C MINUS
CHUGAEV HDL-C)
n = 77

Value (in mg/dL)	Frequency	Percent		
-43.00	1	1		
-42.00	1	1		
-40.00	1	1		
-29.00	1	1		
-28.00	2	3		
-26.00	2	3		
-25.00	1	1		
-24.00	1	1		
-23.00	1	1		
-19.00	1	1		
-18.00	1	1		
-17.00	1	1		
-16.00	1	1		
-15.00	3	4		
-13.00	1	1		
-12.00	1	1		
-11.00	1	1		
- 8.00	1	1		
- 7.00	1	1		
- 6.00	4	5		
- 4.00	3	4		
- 3.00	1	1		
- 2.00	2	3		
- 1.00	1	1		
.00	5	6	Mean	.286
1.00	4	5	SD	19.144
2.00	1	1		
3.00	2	3		
4.00	3	4		
5.00	1	1		
6.00	2	3		
7.00	2	3		
8.00	1	1		
11.00	2	3		
12.00	1	1		
14.00	2	3		
17.00	1	1		
18.00	3	4		
19.00	1	1		
20.00	1	1		
21.00	1	1		
22.00	1	1		
24.00	2	3		
26.00	2	3		
27.00	2	3		
28.00	1	1		
47.00	1	1		
59.00	1	1		

TABLE VIII

FREQUENCY DISTRIBUTION FOR DIFFERENCE
(ALLAIN-TRINDER LDL-C MINUS
CHUGAEV LDL-C)
n = 77

Value (in mg/dL)	Frequency	Percent		
-48.00	1	1		
-45.00	1	1		
-33.00	1	1		
-29.00	1	1		
-26.00	2	3		
-25.00	1	1		
-24.00	2	3		
-23.00	4	5		
-21.00	3	4		
-20.00	1	1		
-19.00	2	3		
-18.00	2	3		
-17.00	1	1		
-16.00	2	3		
-15.00	1	1		
-14.00	2	3		
-11.00	1	1		
-10.00	1	1		
- 9.00	3	4		
- 8.00	1	1		
- 7.00	3	4		
- 6.00	2	3		
- 5.00	1	1		
- 4.00	1	1	Mean	-3.519
- 3.00	3	4	SD	18.387
- 2.00	1	1		
- 1.00	1	1		
.00	1	1		
1.00	2	3		
2.00	1	1		
3.00	1	1		
4.00	1	1		
6.00	2	3		
7.00	1	1		
8.00	3	4		
10.00	1	1		
12.00	2	3		
13.00	1	1		
14.00	2	3		
15.00	3	4		
18.00	1	1		
19 00	1	1		
20.00	1	1		
21.00	1	1		
24.00	1	1		
25.00	1	1		
28.00	1	1		
29 00	1	1		
30.00	1	1		
35.00	1	1		
37 00	1	1		

Discussion of Results

This study compared the results from the Chugaev method of directly measuring the LDL-C fraction of serum cholesterol with the results obtained by Roche Biomedical Laboratories, which used the Allain-Trinder enzymatic method of calculating LDL-C levels. The values determined by both methods for TC and LDL-C were significantly correlated. Since LDL-C was the primary focus of the study, these results are encouraging. As reported by the Laboratory Standardization Panel (LSP) of the National Cholesterol Education Program (NCEP), serious inaccuracies exist in the measured amounts of TC in human serum reference standards (28). Considering only one trial was performed by each method, the similarities in values are noteworthy. Without reproducibility studies, it is not possible to comment on the accuracy or bias of the measurements of either method. More recent work on reproducibility with the Chugaev method is showing promising results (35).

It was expected that the TC values would correlate because E at 518 is calculated from Roche Laboratories numbers. The fact that it does so for so many individuals attests to the fact that the Chugaev method is a valid and reliable method. The good correspondences between the population means is further support for the model used to calculate the fractions.

In the course of the investigation, the values determined for HDL-C and VLDL-C by the two methods were also

compared. The values determined for HDL-C and VLDL-C did not correlate significantly. Although the reasons for this outcome cannot be explained totally, there are several possible explanations.

The estimation technique used by Roche Laboratories relies on the accuracy of the cholesterol and TG assays, the HDL-C precipitation, and the mathematical formula used to estimate the VLDL-C concentration. The accuracy of the Friedewald formula, and therefore the estimation of LDL-C is particularly dependent upon the validity of the assumption that VLDL-C can be estimated by TG/5. DeLong et al (12) found that in fasting samples this has been found to be approximately so when TG value does not exceed 400 mg/dL, but in some circumstances the expression 0.16 x TG leads to a more accurate estimate of VLDL-C, and thereby calculation of LDL-C. A study by McNamara et al (36) compared several VLDL-C estimation methods (TG/4 - TG/8). No single best estimation factor emerged, but use of the factors TG/5 to TG/6 generally yielded the highest percentages. In light of evidence that TG/5 is not always an accurate estimate of VLDL-C, it is possible that the values determined by Roche Laboratories for this fraction are not accurate.

HDL-C measurements require two kinds of manipulations: the isolation of the HDL-C containing fraction from plasma or serum, and then the measurement of cholesterol in this fraction. Some of the analytic variability encountered in HDL-C measurement is related to the difficulty of the

precipitation step employed to obtain a pure sample of HDL-C. This variability is sufficiently great that some authors have suggested that HDL-C measured in an individual patient may not be useful in the primary assessment of risk or change in risk after institution of therapy (6). In the method used by Roche Laboratories, LDL-C and VLDL-C were precipitated from serum with dextran sulfate. Cholesterol remaining in the supernatant solution can be considered to represent HDL-C, if sedimentation of LDL-C and VLDL-C is complete and no HDL-C has precipitated. The cholesterol content of the supernate, and hence the HDL-C, was measured by the enzymatic method of Allain-Trinder with the Olympus Model 5031 analyzer. It is possible, therefore, that differences in the values determined for HDL-C may have resulted because precipitation of LDL-C and VLDL-C in the Allain-Trinder method was not selective or complete.

A striking feature of the HDL-C and VLDL-C values is the closeness of the means without significant correlation. The frequency distributions for these fractions provide an explanation for this observation. For each individual comparison, there are some extremely wide variations of values. For example, the mean difference between HDL-C values was .286, but the individual differences ranged from −43.00 to +59.00.

Approximately 98 percent of all laboratories now participating in the College of American Pathologists proficiency testing survey report the use of enzymatic

procedures (56) used by Roche Laboratories. Although not without limitations, this procedure is reasonably reliable, and represents a well-accepted method. It was expected that the values determined by the Chugaev method would not be significantly different from the values determined by the Allain-Trinder enzymatic method. It is clear from the excellent TC and LDL-C correlations that the chemistry of Chugaev method is able to discriminate among the three cholesterol fractions in a single experimental measurement; that the three fractions are being determined quantitatively; and that the simple mathematical model works. The coefficients in the mathematical model described in Chapter 3 were manipulated to come as close to the values determined by Roche Laboratories as possible. Once pure samples of all fractions are available and measured, the conventional Beer's law calibration curves of A vs. concentration of fraction can be used to give the nine E coefficients without resorting to an empirical fit.

There are also several practical advantages to the Chugaev method over the enzymatic method, including: (1) a smaller volume of blood is required for a full lipid profile; (2) the three fractions are measured, and in a direct manner; (3) only one test rather than three is required to determine LDL-C.

CHAPTER V

SUMMARY, FINDINGS, CONCLUSIONS

AND RECOMMENDATIONS

Summary

The guidelines recently published by the NCEP for the detection, evaluation, and treatment of high blood cholesterol in adults emphasize the reduction of high levels of LDL-C. Reasonably accurate determination of LDL-C is an important aim in view of the significance of this measure as a risk factor for coronary heart disease. This study compared the Chugaev method for direct measurement of LDL-C with the well-accepted Allain-Trinder enzymatic method in which LDL-C levels are calculated from measured total, VLDL-C, and HDL-C levels.

The data collected in the study were analyzed by the following methods:

1. Pearson r between the pairs of scores for each dependent variable was calculated.

2. The percent of each fraction to total cholesterol for each subject and the average percent for each fraction was calculated.

3. The values from one method (Chugaev) were subtracted from the values of the other method (Allain-Trinder) and the differences were averaged.

Findings

Based on the hypothesis stated and the limits of this study, the data yielded the following findings:

1. There was no significant difference in the values for TC and LDL-C as determined by the two methods.

2. Although the mean values for VLDL-C and HDL-C levels measured by both methods for a population of 77 volunteers were in excellent agreement, significant differences occurred between the levels determined for individual members of the population. The statistical significance of this result is not clear at this time.

Conclusions

In consideration of the results the conclusion that the Chugaev method is able to measure LDL-C as accurately as the widely accepted enzymatic method seems warranted.

Recommendations

Based on the data collected in this study, it is evident that additional research is needed to establish the Chugaev method as a viable alternative for the well established enzymatic method.

Until pure fraction samples become available it is not possible to address the accuracy in the measurements. In the meantime, extensive reproducibility studies need to be made in order to compare the relative precisions attainable by both procedures. These investigations should include various population groups, by age, race, and gender. There is good reason to believe that high triglyceride levels are not a deterent to direct measurement of VLDL-C by the Chugaev method and patients with hypertriglyceridemia might benefit greatly from additional lipid profile information.

The following recommendations are also presented as a result of this study as means of refining the Chugaev procedure:

1. Increase the volume of serum to reduce inaccuracies due to measurement error.

2. Improve care in storage to keep reagents dry. If they get wet in storage, the reagent mix is altered and they produce a different chemical reaction.

3. Run a baseline before every measurement to reduce error due to instrument drift.

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APPENDIXES

APPENDIX A

INFORMED CONSENT

OSU WELLNESS CENTER
INFORMED CONSENT FORM

BLOOD TESTING

Explanation of Test

The blood test you are about to undergo is part of the Oklahoma State University Wellness Program. The test includes selected blood variables analyzed from the fingerstick method or from a venous sample.

It will be determined, prior to testing, that this test is appropriate and safe for you. All testing will be conducted by trained personnel and procedures will be explained to your satisfaction at the outset.

Possible Risks

The potential risks associated with the venipuncture/fingerstick are (1) Venipuncture/fingerstick may cause some pain or discomfort. The exact amount, if any, will be dependent upon individual preconceptions and pain threshold levels (2) Possible hematoma (bruising) at the venipuncture/fingerstick site following the procedure. The occurrence or non-occurrence will be dependent upon bleeding/coagulation times and adherence to instructions pertaining to holding a cotton ball against the venipuncture/fingerstick site, with pressure, for five minutes following extraction of the needle or following the fingerstick. (3) Slight risk of infection. Any break in the integrity of the skin is associated with a small degree of risk infection. However, if directions are followed the risk is very small.

Consent by Subject

The information which is obtained will be treated as privileged and confidential and will not be released or revealed to anyone without your express consent. Information will, however, be treated in an aggregate manner to provide group information. In addition, if indicated, a small amount of the blood drawn may be used for research in alternative cholesterol testing.

I have read the foregoing, I understand it, and any questions which may have occurred to me have been answered to my satisfaction.

Date _____

Subject Signature _____

Witness Signature _____

APPENDIX B

RAW DATA

RAW DATA
(in mg/dL)

SUBJECT NO.	TC	VLDL-C	LDL-C	HDL-C
01 ALLAIN-TRINDER	225	36	152	37
CHUGAEV	215	27	158	30
02 ALLAIN-TRINDER	140	12	76	51
CHUGAEV	143	8	85	51
03 ALLAIN-TRINDER	229	32	161	36
CHUGAEV	236	28	153	55
04 ALLAIN-TRINDER	215	29	152	33
CHUGAEV	243	19	175	48
05 ALLAIN-TRINDER	239	41	167	31
CHUGAEV	248	10	166	71
06 ALLAIN-TRINDER	162	19	106	36
CHUGAEV	154	6	96	52
07 ALLAIN-TRINDER	218	20	140	57
CHUGAEV	211	6	132	72
08 ALLAIN-TRINDER	184	15	136	32
CHUGAEV	195	25	132	38
09 ALLAIN-TRINDER	135	18	76	40
CHUGAEV	137	14	79	44
10 ALLAIN-TRINDER	191	16	141	34
CHUGAEV	183	20	112	51
11 ALLAIN-TRINDER	229	32	161	35
CHUGAEV	226	11	162	53
12 ALLAIN-TRINDER	250	39	174	36
CHUGAEV	275	8	188	79
13 ALLAIN-TRINDER	184	18	83	82
CHUGAEV	202	7	131	64

SUBJECT NO.		TC	VLDL-C	LDL-C	HDL-C
14	ALLAIN-TRINDER	157	16	97	43
	CHUGAEV	175	8	111	56
15	ALLAIN-TRINDER	220	16	149	55
	CHUGAEV	244	28	135	81
16	ALLAIN-TRINDER	154	8	101	44
	CHUGAEV	167	11	104	52
17	ALLAIN-TRINDER	224	20	162	41
	CHUGAEV	232	26	182	24
18	ALLAIN-TRINDER	254	35	138	81
	CHUGAEV	268	16	183	69
19	ALLAIN-TRINDER	253	42	176	34
	CHUGAEV	270	8	186	76
20	ALLAIN-TRINDER	223	27	160	35
	CHUGAEV	226	21	164	41
21	ALLAIN-TRINDER	232	29	151	52
	CHUGAEV	239	13	169	58
22	ALLAIN-TRINDER	266	15	212	38
	CHUGAEV	259	30	193	36
23	ALLAIN-TRINDER	235	12	151	72
	CHUGAEV	232	20	168	44
24	ALLAIN-TRINDER	218	36	150	31
	CHUGAEV	227	9	158	59
25	ALLAIN-TRINDER	159	6	103	49
	CHUGAEV	152	12	105	35
26	ALLAIN-TRINDER	247	12	183	51
	CHUGAEV	244	32	181	31
27	ALLAIN-TRINDER	159	12	92	55
	CHUGAEV	165	9	115	41
28	ALLAIN-TRINDER	166	10	115	40
	CHUGAEV	171	10	122	39
29	ALLAIN-TRINDER	234	14	166	54
	CHUGAEV	231	16	171	43
30	ALLAIN-TRINDER	238	24	151	63
	CHUGAEV	247	13	172	62

SUBJECT NO.		TC	VLDL-C	LDL-C	HDL-C
31	ALLAIN-TRINDER	244	22	170	52
	CHUGAEV	256	24	199	34
32	ALLAIN-TRINDER	160	14	111	35
	CHUGAEV	163	9	108	46
33	ALLAIN-TRINDER	181	12	103	66
	CHUGAEV	184	12	128	45
34	ALLAIN-TRINDER	169	11	98	60
	CHUGAEV	177	14	122	41
35	ALLAIN-TRINDER	171	8	123	39
	CHUGAEV	170	16	115	39
36	ALLAIN-TRINDER	230	41	150	39
	CHUGAEV	244	14	166	64
37	ALLAIN-TRINDER	260	17	196	46
	CHUGAEV	260	23	190	46
38	ALLAIN-TRINDER	243	41	148	53
	CHUGAEV	249	2	167	79
39	ALLAIN-TRINDER	164	18	96	49
	CHUGAEV	165	9	112	45
40	ALLAIN-TRINDER	178	13	118	47
	CHUGAEV	181	13	127	41
41	ALLAIN-TRINDER	204	9	125	69
	CHUGAEV	202	14	146	42
42	ALLAIN-TRINDER	187	12	116	59
	CHUGAEV	194	16	142	37
43	ALLAIN-TRINDER	215	23	148	44
	CHUGAEV	226	25	130	72
44	ALLAIN-TRINDER	253	18	179	55
	CHUGAEV	252	30	190	31
45	ALLAIN-TRINDER	256	22	192	41
	CHUGAEV	238	32	164	41
46	ALLAIN-TRINDER	193	17	122	53
	CHUGAEV	200	14	129	57
47	ALLAIN-TRINDER	165	9	93	62
	CHUGAEV	168	12	119	36

SUBJECT NO.	TC	VLDL-C	LDL-C	HDL-C
48 ALLAIN-TRINDER	185	21	113	50
CHUGAEV	177	10	112	56
49 ALLAIN-TRINDER	214	13	138	62
CHUGAEV	223	24	161	38
50 ALLAIN-TRINDER	229	28	159	41
CHUGAEV	237	20	180	37
51 ALLAIN-TRINDER	180	43	115	21
CHUGAEV	159	14	95	50
52 ALLAIN-TRINDER	215	38	146	30
CHUGAEV	197	21	131	45
53 ALLAIN-TRINDER	228	19	170	39
CHUGAEV	197	32	133	32
54 ALLAIN-TRINDER	198	51	104	42
CHUGAEV	186	13	119	54
55 ALLAIN-TRINDER	249	21	144	83
CHUGAEV	233	32	177	24
56 ALLAIN-TRINDER	183	19	122	41
CHUGAEV	158	16	98	43
57 ALLAIN-TRINDER	214	43	144	27
CHUGAEV	207	18	138	51
58 ALLAIN-TRINDER	169	20	111	37
CHUGAEV	165	19	117	29
59 ALLAIN-TRINDER	189	29	92	67
CHUGAEV	179	8	116	56
60 ALLAIN-TRINDER	247	44	168	34
CHUGAEV	238	28	177	34
61 ALLAIN-TRINDER	181	18	118	44
CHUGAEV	175	16	121	38
62 ALLAIN-TRINDER	253	36	168	48
CHUGAEV	249	33	187	30
63 ALLAIN-TRINDER	146	16	98	32
CHUGAEV	132	12	83	36
64 ALLAIN-TRINDER	164	14	112	37
CHUGAEV	156	19	99	38

SUBJECT NO.	TC	VLDL-C	LDL-C	HDL-C
65 ALLAIN-TRINDER	177	25	112	39
CHUGAEV	161	15	100	46
66 ALLAIN-TRINDER	236	29	173	33
CHUGAEV	215	28	159	28
67 ALLAIN-TRINDER	152	10	94	48
CHUGAEV	133	13	69	51
68 ALLAIN-TRINDER	180	16	111	53
CHUGAEV	160	23	111	26
69 ALLAIN-TRINDER	152	15	96	40
CHUGAEV	118	10	66	42
70 ALLAIN-TRINDER	133	16	65	52
CHUGAEV	134	20	88	26
71 ALLAIN-TRINDER	184	11	130	42
CHUGAEV	172	19	115	38
72 ALLAIN-TRINDER	186	21	128	36
CHUGAEV	177	21	121	35
73 ALLAIN-TRINDER	179	10	108	61
CHUGAEV	164	25	126	14
74 ALLAIN-TRINDER	124	16	62	45
CHUGAEV	100	8	50	42
75 ALLAIN-TRINDER	181	29	116	35
CHUGAEV	142	4	81	58
76 ALLAIN-TRINDER	220	24	153	42
CHUGAEV	198	25	132	41
77 ALLAIN-TRINDER	179	25	116	37
CHUGAEV	175	18	123	34

VITA

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