METHODS AND PRINCIPLES

Cholesterol

Introduction

The standard procedures for cholesterol and triglyceride measurements are the Monotest Cholesterol procedure (1) and the GPO Triglyceride procedure (2) of Boehringer Mannheim. Both of these tests are adapted to automatic analysis using the Hitachi 911 Analyzer. By using these reagents and instrumentation we have demonstrated that measurements meet the criteria of precision and accuracy set forth by CDC. A number of factors are involved in the interference of the enzymatic determination of plasma lipids (3,4). Among common drugs, only ascorbic acid and L-methyldopa produced borderline negative interference at therapeutic plasma concentrations. In Trinder systems (5), bilirubin has been reported to interact in the peroxidase-catalyzed reaction by competing for hydrogen peroxide, which results in lower lipid levels (6). The inclusion of hexacyanoferrate (II) into the reagent mixture minimizes this chemical interference, and bilirubin concentrations of up to 15 mg/dL did not produce significant changes in results. Positive interference may be caused by hemoglobin, which acts as a pseudoperoxidase. However, no distortion of results was observed by concentrations of up to 2 g/dL.

Since the proper functioning of the Hitachi 911 Analyzer is essential for the timely analysis of samples, a detailed maintenance program is in operation, which includes a service contract with 24 hour response time of a field engineer.

Automated Determination of Cholesterol

Principal

Cholesteryl esters are hydrolyzed to free cholesterol and fatty acids by cholesteryl esterase. The free cholesterol produced and that already present in the sample is oxidized by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. Peroxidase catalyzes the reaction of hydrogen peroxide, 4-aminoantipyrine, and phenol (or a substituted phenol) to produce a quinoneimine dye, with maximum absorbance at 500 nm. The absorbance readings at this wavelength are proportional to the concentration of total cholesterol in the sample (1).

Materials

Equipment and Supplies
- Boehringer Mannheim/Hitachi 911
- Gilson pipetman
- Sample cups
- Cells for Hitachi 911.

The Hitachi 911 analyzer is a totally self-contained Computerized, Programmable, Discrete, Selective, Sequential, fully automated Chemistry Analyzer. It is composed of two hardware units:
- The analytical unit and the control unit. The 911 analyzer uses several operational systems to perform required functions.

Cholesterol Reagent:

The Cholesterol / HP reagent is supplied from Boehringer Mannheim Corporation. The reagent is automatically shipped to the Laboratory once a month. “High Performance” is the acceptable reagent grade. The ingredients for the Cholesterol Reagent are as follows:
0.40 mmol 3,4-Dichlorophenol
0.60 mmol Phenol
0.10 mmol 4-Aminophenazone
≥ 25 U Cholesterol Oxidase (EC 1.1.3.6: Nocardia erythropolis; 25°C)
≥ 40 U Cholesterol Esterase (EC 3.1.1.13: microorganism; 25°C)
≥ 20 U POD (EC 1.11.1.1.7; horse radish; 25°C)
Non Reactive Ingredients: Buffer, Stablizers, and Preservatives

Additional materials that are required are an automated clinical chemistry analyzer, Precical Calibrator Serum, and a solution of 0.9% NaCl.

Reagent Preparation

Cholesterol / HP reagent comes prepackaged in two forms. One is a premeasured powder that is reconstituted in its bottle with 100 mL of distilled or deionized water from the tank attached to the Hitachi 911. When mixed gently it is ready for use in 10 minutes. The other form is a ready-to-use premade solution, which requires no reconstitution. The degree of accuracy required for measuring reagents is a maximum error for glassware of ± 5%. It is necessary to use Class A Volumetric Glassware to meet performance guidelines. Reagents should only be used according to the manufacturer’s specific guidelines for this specific procedure.

Storage Requirements

Reagent is stored in the bottle that it arrives in from the supplier. It should be stored at 2-8°C. Refer to the labels on the box and bottles for expiration dates on unopened components. Once opened the reagent should be put into the analyzer. The analyzer will alert the tech when the reagent has passed its shelf life. Reconstituted reagent is stable for up to 4 weeks at 2-12°C or 7 days at 20-25°C when protected from light or contamination by microorganisms. Reagent bottles should be labeled with the following: substance (cholesterol reagent), lot number, date of preparation, expiration date, special storage instructions. In the event that the reagent does not perform within set parameters, eliminate other potential problems, prepare a new bottle of reagent and recalibrate. If the problem persists, call the Manufacturer’s technical support.

Calibration

The Hitachi 911 automatically performs calibration on line. Calibrators are manufactured by Boehringer Mannheim and are prepared according to manufacturer’s insert

Storage Requirements

Store unopened Precical Calibrator Serum at 2-8 degrees Celsius or 20-25 degrees Celsius. Do Not Freeze! Each vial bears the expiration date on the label. No evidence of vacuum in vial upon opening is an indication of possible deterioration of unreconstituted Precical Calibrator Serum. If there is any evidence of coloration, cloudiness or turbidity in Precical Diluent the vial should be discarded.

Reconstituted Precical Calibrator Serum is stable for two days when stored securely stoppered and protected from light at 28 degrees Celsius.

1Boehringer Mannheim Cholesterol /HP Application, pp. 2.
Opened Precical Calibrator Serum, Precitrol-N and Precitrol-A should be labeled with date of opening and the initials of the person preparing them.

Controls and Standards

All standards and controls are tested in duplicate for quality control purposes. Various suppliers provide the controls that are used. Boehringer Mannheim provides the Precitrol-N, for normal control, the Precitrol-A, for abnormal control, and the Precinorm-L, for normal control that is tested twice each month. Solomon Park provides an additional standard (Sercal) which is tested daily for the Cholesterol and Triglyceride levels. Pacific Biometrics provides three additional controls: Lot A, B, and Lot C. These three controls are tested daily for cholesterol, triglyceride, HDL and Creatinine. Additionally, Incstar provides standards and controls for the Apolipoprotein A-1, Apolipoprotein B and Lipoprotein(a) tests that are performed when requested.

Calculations

The Hitachi 911 does all Calculations.

Analytical Procedure

Hitachi 911

After performing daily maintenance on the Hitachi 911 pipette Precical calibrator, Precitrol-n, Precitrol-A and Precimat standard into sample cups and place in appropriate positions on the standard and control section on the sample disk. Program the Hitachi 911 to perform required calibrations for tests to be calibrated. Test that have 3 or more calibrators always get a full calibration. Pipette a minimum of 0.5mL of each plasma specimen into a sample cup (without top). Specimens are numbered 00001-800. following the loading of specimens, the number of specimens and quality control standards are programmed into the cholesterol program. Sample volume 3μl and reagent volume 300μl. Incubation temperature is 37°C, and running time is 6 min. Cholesterol content of samples is calculated by the microprocessor of the instrument using the Autoblank. Endpoint Mode.

References

1. Lieberman C. Ber. 1885;18:1803
2. Burchard H. Chem Zentr. 1890;61:25
Triglycerides

Principal

Triglycerides are hydrolyzed to glycerol and fatty acids by lipase. The glycerol formed is then phosphorylated in the presence of ATP. Oxidation of the resulting glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide is catalyzed by glycerol phosphate oxidase. An intense red chromogen is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate with hydrogen peroxide. The absorbance readings at 510 nm are proportional to the concentration of total glycerol content (free and esterified) in the samples (1).

Materials

Equipment and Supplies
- Boehringer Mannheim/Hitachi 911
- Gilson pipetman
- Sample cups
- Cells for Hitachi 911.

The Hitachi 911 analyzer is a totally self-contained Computerize, Programmable, Discrete, Selective, Sequential, fully automated Chemistry Analyzer. It is composed of two hardware units:
- The analytical unit and the control unit. The 911 analyzer uses several operational systems to perform required functions.

Reagents

The Triglyceride reagent is supplied from Boehringer Mannheim Corporation (Cat. No. 7474029). It is a mixture of R1 Buffer, 4-Chlorophenol and enzymes. The reagent is automatically shipped to the Laboratory once a month with a standing order. “High Performance” is the acceptable reagent grade. The ingredients for the Triglyceride Reagent are as follows:

3.5 mmol/L 4-Chlorophenol
≥0.5 mmol/L ATP
0.35 mmol/L 4-Aminophenazone
≥3 U/mL Lipase (EC 3.1.1.13; pseudomonas species; 25°C)
≥2.5 U/mL Glycerol phosphate oxidase (EC 1.1.3.21)
≥0.2 U/mL Glycerokinase (EC 1.1.3.21; Candida mycoderma; 25°C)
≥0.15 U/mL Peroxidase (EC 1.1.1.7; horseradish; 25°C)
Nonreactive ingredients: Buffer, preservative, detergent

Additional materials that are required are an automated clinical chemistry analyzer, Precical Calibrator Serum, and a solution of 0.9% NaCl.

Reagent Preparation

Triglycerides/GPO reagent can mixed in the shipping bottles. Connect Bottle 1 containing buffer and 4-Chlorophenol to Bottle 1a containing enzymes using the enclosed adapters. Mix by gentle inversion. Disconnect Bottle 1a and connect the second Bottle 1a. Mix again by gentle inversion until the lyophilizate is completely dissolved in the buffer. Reagent should only be used according to the manufacturer’s specific guidelines for this specific procedure.

Storage Requirements
Reagent is stored in the shipping bottles. It should be stored at 2-8°C. Refer to the labels on the box and bottles for expiration dates on unopened components. Once opened the reagent should be put on board the analyzer. The analyzer will alert the tech when the reagent has passed its shelf life. Reconstituted reagent is stable for up to 14 days at 2-12°C or 2 days at 15-25°C when protected from light or contamination by microorganisms. Reagent bottles should be labeled with the following: substance (triglyceride reagent), lot number, date of preparation, expiration date, special storage instructions (Initial of Technician). In the event that the reagent does not perform within set parameters, eliminate other potential problems, prepare a new bottle of reagent and recalibrate. If the problem persists, call the Manufacturer’s technical support.

**Calibration**

The Hitachi 911 automatically performs calibration on line. Calibrators are manufactured by Boehringer Mannheim and are prepared according to manufacturer’s insert.

**Storage Requirements**

- Store unopened Precical Calibrator Serum at 2-8 degrees Celsius or 20-25 degrees Celsius. Do Not Freeze! Each vial bears the expiration date on the label.
- No evidence of vacuum in vial upon opening is an indication of possible deterioration of unreconstituted Precical Calibrator Serum.
- If there is any evidence of coloration, cloudiness or turbidity in Precical Diluent the vial should be discarded.

Reconstituted Precical Calibrator Serum is stable for two days when stored securely stoppered and protected from light at 28 degrees Celsius.

Opened Precical Calibrator Serum, Precitrol-N and Precitrol-A should be labeled with date of opening and the initials of the person preparing them.

**Controls and Standards**

All standards and controls are tested in duplicate for quality control purposes. Various suppliers provide the controls that are used. Boehringer Mannheim provides the Precitrol-N, for normal control, the Precitrol-A, for abnormal control, and the Precinorm-L, for normal control that is tested twice each month. Solomon Park provides an additional standard (Sercal) which is tested daily for the Cholesterol and Triglyceride levels. Pacific Biometrics provides three additional controls: Lot A,B and Lot C. These three controls are tested daily for cholesterol, triglyceride and HDL. Additionally, Incstar provides standards and controls for the Apolipoprotein A-1, Apolipoprotein B and Lipoprotein(a) tests that are performed when requested.

**Calculations**

The Hitachi 911 does all Calculations.
Analytical Procedure

Hitachi 911

After performing daily maintenance on the Hitachi 911 pipette Precical calibrator, Precitrol-n, Precitrol-A and Precimat standard into sample cups and place in appropriate positions on the standard and control section on the sample disk. Program the Hitachi 911 to perform required calibrations for tests to be calibrated. Test that have 3 or more calibrators always get a full calibration. Pipette a minimum of 0.5mL of each plasma specimen into a sample cup (without top) Specimen are numbered 00001-800. following the loading of specimens, the number of specimens and quality control standards are programmed into the cholesterol program. Sample volume 3μl and reagent volume 300μl. Incubation temperature is 37°C, and running time is 6 min. Triglyceride content of samples is calculated by the microprocessor of the instrument using the Autoblank, Endpoint Mode.

References

Triglycerides/GPO:

Measurement of HDL-Cholesterol

**Principal**

Methods to quantify plasma levels of HDL include various kinds of ultracentrifugation (1-3), electrophoretic techniques (4), and procedures which rely on precipitation of lipoproteins other than HDL (5). While the classical ultracentrifugation techniques are impractical for analyzing such large numbers of samples as required in this program, precipitation techniques have been proven useful in previous epidemiological studies (6-9). In the majority of these studies, VLDL and LDL were precipitated by heparin and Mn2+, and the cholesterol of HDL remaining in solution was measured by a modified Lieberman Burchard method. The use of heparin and Mn2+ is, however, inadequate when cholesterol is determined by enzymatic procedures, since Mn2+ interferes in the determination (10). To overcome this drawback, Mg2+ and dextran sulfate was used and evaluated to precipitate VLDL and LDL (5,11). The method was found highly reliable and reproducible, but revealed, on the average, 5% lower plasma levels of HDL-cholesterol, when compared with the LRC-procedure (11). The explanation for this negative bias may be that a more complete precipitation of Apo B-containing lipoproteins (11) and precipitation of Apo E-containing lipoproteins (12) had occurred. The supernate contained less than 10% of total apo(a) immunoreactivity (13). These data are similar to a previous study in which about 10% of apo(a) immunoreactivity was found in the lipoprotein-free fraction subsequent to sequential ultracentrifugation (14). Thus about 10% of apo(a) is associated with little or no lipid.

Mixtures of dextran sulfate (molecular weight 50,000), an analog of heparin, and magnesium ions (Mg2+) cause precipitation of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) while the high density lipoproteins (HDL) remain in solution. Precipitated lipoproteins are separated by centrifugation, and the supernate containing HDL is analyzed for cholesterol content.

**Materials**

**Equipment and Supplies**
- Boehringer Mannheim/Hitachi 911
- Gilson pipetman
- Sample cups
- Cells for Hitachi 911.

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**Reagents**

The HDL-Cholesterol reagent is supplied from Boehringer Mannheim Corporation. The reagent is automatically shipped to the Laboratory once a month with a standing order. “High Performance” is the acceptable reagent grade. The precipitant for the reagent is a combination of:

- 0.55 mmol/L Phosphotungstic acid
- 25 mmol/L Magnesium chloride
- 75 mmol/L PIPES buffer, pH 6.8
- 10 mmol/L Mg2+
- 0.2 mmol/L Sodium cholate
- 0.15 mmol/L 4-Aminophenazone
- ≥4.2 mmol/L Phenol
- 1% Fatty alcohol-polyglycol ether
≥0.5 U/mL Cholesterol esterase (EC 3.1.1.13; *pseudomonas* species; 25°C)
≥0.15 U/mL Cholesterol oxidase (EC 1.1.3.6; *E. coli*; 25°C)
≥0.25 U/mL Peroxidase (POD) (EC 1.11.1.7; horseradish; 25°C)

Nonreactive Ingredients: Buffer, stabilizers, preservative

Additional materials that are required are an automated clinical chemistry analyzer, Precimat 50mL Cholesterol Calibrator, and a solution of 0.9% NaCl.

**Reagent Preparation**

HDL-Cholesterol reagent comes in two bottles; one containing the precipitant and the other containing the working solution. The precipitant is ready for use. The working solution should be mixed by gently swirling before use. Reagent should only be used according to the manufacturer's specific guidelines for this specific procedure.

**Storage Requirements**

Reagent and precipitant are stored in the shipping bottles. Reagent should be stored at 2-8°C. The precipitant should be stored at 15-25°C. Refer to the labels on the box and bottles for expiration dates on unopened components. Once opened the reagent and precipitant should be put on board the analyzer. The analyzer will alert the tech when the reagent or precipitant has passed its shelf life. Reconstituted reagent is stable for up to 28 days at 2-12°C or 7 days at 15-25°C when protected from light or contamination by microorganisms. Precipitant will remain stable until the expiration date on the bottle when stored at 15-25°C. Reagent and precipitant bottles should be labeled with the following: substance (HDL-Cholesterol reagent or precipitant), lot number, date of preparation, expiration date, special storage instructions. In the event that the reagent does not perform within set parameters, eliminate other potential problems, prepare a new bottle of reagent and recalibrate. If the problem persists, call the Manufacturer's technical support.

**Calibration**

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**Storage Requirements**

Store unopened Precical Calibrator Serum at 2-8 degrees Celsius or 20-25 degrees Celsius. Do Not Freeze! Each vial bears the expiration date on the label.

No evidence of vacuum in vial upon opening is an indication of possible deterioration of unreconstituted Precical Calibrator Serum.

If there is any evidence of coloration, cloudiness or turbidity in Precical Diluent the vial should be discarded.

Reconstituted Precical Calibrator Serum is stable for two days when stored securely stoppered and protected from light at 208 degrees Celsius.

Opened Precical Calibrator Serum, Precitrol-N and Precitrol-A should be labeled with date of opening and the initials of the person preparing them.

**Controls and Standards**

All standards and controls are tested in duplicate for quality control purposes. Various suppliers provide the controls that are used. Boehringer Mannheim provides the Precitrol-N, for normal control, the Precitrol-A, for abnormal control, and the Precinorm-L, for normal control that is tested twice each month. Solomon Park provides an additional standard (Sercal) which is tested daily for the Cholesterol and Triglyceride levels. Pacific Biometrics provides three additional
controls: Lot A, B and Lot C. These three controls are tested daily for cholesterol, triglyceride and HDL. Additionally, Incstar provides standards and controls for the Apolipoprotein A-1, Apolipoprotein B and Lipoprotein(a) tests that are performed when requested.

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Analytical Procedure

Hitachi 911

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References