New System for Automated Extraction and Simultaneous Determination of Serum Cholesterol and Triglycerides

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We describe an automated system for serum lipid extraction and simultaneous determination of cholesterol and triglycerides, with use of continuous-flow equipment. A sample volume of 100 μl of serum is required, and samples are processed at the rate of 20 per hour, which may be increased with slight loss in precision. Time from sample pickup to recorder readout is about 25 min. The system makes use of established colorimetric reactions, and the values obtained agree with ranges currently reported in the literature. Correlation coefficients for results of the automated and manual methods were 0.98 for cholesterol and 0.99 for triglycerides, and the day-to-day coefficients of variation were 1.8% for cholesterol (1 SD = 34 mg/liter) and 3.4% for triglycerides (1 SD = 37 mg/liter). The small sample volume, precision, accuracy, speed, and comparative economy of reagents make this system particularly suitable for multiphasic screening, pediatrics, and small-animal research.

Additional Keyphrases: screening • pediatric chemistry • continuous-flow analysis • intermethod comparison

Although numerous published reports describe methods for determination of cholesterol and triglycerides (triacylglycerols) in blood, none has provided a system for automated extraction and simultaneous determinations by use of established colorimetric reactions. For several years we have been involved in the problems of mechanization of lipid analyses. We present here the results of our efforts, a totally automated system that requires only 100 μl of serum and little specialized equipment other than a basic continuous-flow system (Technicon Instruments Corp., Tarrytown, N.Y. 10591). Samples are analyzed at the rate of 20 per h, with about 25 min elapsed time from sample pickup to recorder readout.

Fig. 1. Simplified flow diagram of the analytical system

Materials and Methods

Overview

Figure 1 shows a simplified flow diagram. The serum sample is aspirated and mixed with the diluent with no air segmentation. The mixture is immediately injected into a segmented stream of solvent, where a single phase is obtained. The sample “disappears” into the solvent as the stream progresses through a mixing coil, and coagulation of the protein is not apparent. After thorough mixing, two phases are obtained by the introduction of dilute acid, at which time the protein reappears in the aqueous (upper) phase as a finely divided coagulum. The two-phase stream immediately passes into a Teflon delay coil and then to a glass separator, where an aliquot of the lower solvent layer is withdrawn for analysis. The remaining liquid and protein phases pass off to waste.

The solvent aliquot is injected into a segmented stream of alcoholic hydroxide and, in the single-phase organic medium, the lipids are quickly hydrolyzed at room temperature. This stream then flows into a Teflon dialyzer, where the glycerol produced by hydrolysis is dialyzed into an aqueous stream. The aqueous stream flows to a glass separator, where an
 aliquot is removed and taken through the Hantzsch reaction for the determination of triglycerides.

The donor solvent stream, emitted from the dialyzer, is washed by the addition of dilute acid to remove hydroxide and alcohol, and then flows to a glass separator, where an aliquot of the lower solvent phase is withdrawn and injected into a segmented stream of preheated acid color-reagent for the determination of cholesterol by the Kiliani reaction with ferric chloride. The manifold flow diagram is shown in Figure 2, and an overall view of the operating system is shown in Figure 3.

This is a closed system, and no chemical vapors are allowed in the working area. All waste lines are fed into Teflon tubing down into a glass sink trap, where all waste is emitted below the water level of the trap. A moderate flow of water is maintained during operation of the system. If local conditions are not designed to permit direct disposal of the waste in the laboratory sink, waste lines can be run to the bottom of a container sufficiently large to allow an adequate water layer to be carried above the emitted waste.

Basic Equipment

Two or more peristaltic pumps are required, depending upon capacity. One of these must be of the "open face" type, in which the manifold tubing and rollers are physically separated from the drive motor and housing, such as the Technicon Model I. The high volatility of the solvent precludes the use of any

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**Fig. 2.** Manifold for extraction and analyses

a. pump No. 1 (open face); b. pump No. 2
enclosed pump where heat from the motor cannot be dissipated away from the manifold.

One dialyzer frame, Technicon Model I.

Two oil heating baths, Technicon Model I with adjustable temperature control. One bath with a 12 m (40 ft) coil and temperature of 60 °C, and one bath with two short coils of seven turns each (3.6 m; 12 ft) and temperature 80 °C.

Two colorimeters, Technicon Model II.

Two interference filters, 550 nm for cholesterol and 412 nm for triglycerides.

Two recorders, or one dual-pen recorder.

One automatic sampler, Technicon Model II-IV. The Model I sampler cannot be used because of large air intake between samples.

Basic fittings and materials are designated in Figure 2a and b.

All chemicals are reagent grade, and water is distilled or de-ionized.

Extraction and Hydrolysis

Diluent solution: To 500 ml of water add 50 ml of absolute methanol and 10 ml of concentrated hydrochloric acid. Dilute to 1 liter with water. Prepare freshly each day.

Solvent solution: Mix 1.5 liter of chloroform and 1.5 liter of absolute methanol. Store in a brown bottle in a freezer. Before use, the bottle is brought to 0 °C, and placed in an ice bath; during use the solvent temperature is maintained at 0 ± 1 °C. Stable indefinitely.

Hydrochloric acid, 5, 10 and 40 ml/liter.

Potassium hydroxide, 50 g/liter, aqueous.

Ethanol, absolute.

Triglyceride Reagents

Ammonium acetate, 2.2 mol/liter, aqueous. Adjust pH to 6.0. Store in brown bottle. Stable indefinitely.

Periodate solution: Dissolve 1.3 g of sodium metaperiodate in 10 ml of water and dilute to 1 liter with ammonium acetate, 2.2 mol/liter. Readjust the pH to 6.0 if necessary. Store in brown bottle. Stable indefinitely.

Rhamnose, 20 g/liter, aqueous. Stable for at least one week. Watch for bacterial contamination.

Acetylated acetone, 5 ml/liter, aqueous. Store in brown bottle. Stable indefinitely.

Cholesterol Reagent

Dissolve 0.825 g of ferric chloride in 2.4 liters of glacial acetic acid. Chill thoroughly in an ice bath. Cautiously add 800 ml of cold, concentrated sulfuric acid. Mix well and allow to come to room temperature. Store in a brown bottle. Mixing at low temperature produces a more stable reagent with less initial color. This reagent darkens with age, but is usable for about a week. While in use, the bottle should have a two-hole stopper with a desiccant tube in the airway.

Standards

Secondary standards must be used because only whole serum can be used in this system. Commercial "aqueous" standards or serum diluted with either physiological saline or bovine albumin solutions cannot be used for a standard curve because the extraction is not comparable to that for whole serum. Instead, human sera are pooled to provide six pools in a range of concentrations. Our standard range is about 1.25–3.0 g of cholesterol per liter and 0.5–3.0 g triglycerides per liter. Initially the concentrations of these pools must be determined with primary standards by a manual technique. These pools are aliquoted and frozen for use as standards; thereafter, successive pools are analyzed in the automated system. Although the accuracy of the system must be monitored with a well-designed quality-control program, the long-term stability and precision of the system obviates the disadvantage of secondary standards.

Procedure

At the beginning of operation the three Acidflex manifold tubes for aliquots are left disconnected from the C4 separators. These separators are temporarily plugged by attaching blocked tubing to the nipples (tie a knot in tubing of small i.d.). Aqueous solutions must never be pumped through the Acidflex manifold tubes.

Place the solvent bottle in a large container and surround the bottle with ice. Start pumping all reagents. Invert the solvent reservoirs to fill with solvent. With reservoirs upright, observe the fluid flow and bubble pattern in the extraction coils. When the two phases are flowing smoothly through the first C4 separator, attach the two YEL/YEL aliquot lines. (The second line, which flows to waste, is necessary to maintain the separation characteristics.) After the hydrolyzed chloroform extract has passed through
the dialyzer and wash coil and the two phases are flowing smoothly through the second C4 separator, attach the GRY/GRY cholesterol aliquot line. Check the lower end of both separators for trapped air bubbles or emulsion. Allow systems to stabilize for at least one hour.

Load the sample wheel, with three wash cups between each sample. Wash cups are filled with the 5 ml/liter hydrochloric acid, as is the constant-volume reservoir. Use a sampler cam of 80 per hour with a 9:1 sample/wash ratio. The wheel cover should always be used, to minimize sample evaporation, and samples should not stand or be resampled over long periods of time. After stable baselines are established, prime and calibrate the system by repeatedly sampling a pool of known concentration. We use 30 mg/liter per line unit, making the recorder span 3.0 g/liter for routine runs.

At the end of the run, when the last sample has been recorded, first disconnect and plug the two C4 separators. Remove the solvent intake line and pump air only. Remove the cholesterol color reagent intake line and pump air only. Pump water through all other reagent lines until the system is washed out. The glass and Teflon extraction coils, sample probe, and line should be flushed with a cleaning solution (1 mol/liter NaOH) and then washed thoroughly with water. This is easily accomplished by placing the probe and diluent pick-up lines in cleaning solution after all solvent has been pumped out of system. The cholesterol reagent and pull-through manifold tubes should be changed daily. All other manifold tubes are changed weekly. The membrane in the dialyzer should be changed once a month.

Notes on Operation

This system is easy to operate and malfunctions are uncommon. The following notes cover those areas that require special attention.

1. The fluid dynamics of this system require a modification of the colorimeter. With unmodified 50-mm flowcell there is intermittent interruption of the lightpath by the passing air bubbles, and we have found it necessary to remove the air segment before the stream enters the flow cell, a simple modification. The waste side of the flow cell debubbler is plugged. A separator (C1) is sleeved directly to the exit port of the cell, which now becomes the entry port. The inlet side of the debubbler is connected to the pull-through line on the pump. The segmented stream enters the short side of C1; the air flows vertically out the top to the waste line; the debubbled stream enters the flowcell in reverse and is pulled through the cell, analogous to the Model I colorimeter.

2. To maintain the necessary bubble pattern during extraction and hydrolysis, all coils, dialyzer head, and pump platen must be placed on the same plane, with a minimum of vertical displacement (Figure 4). Abrupt changes in relative height will cause the solvent segments to slide past the air segments.

3. The amount of air and its introduction into the system are critical. The initial air segmentation must be introduced into the solvent stream before the diluted sample is injected. The sample and diluent are mixed, without air, immediately before injection into the segmented solvent stream. Excess air will cause evaporative changes in fluid volumes and cause premature protein precipitation. However, the minute air bubbles picked up by the probe as it travels from the sampler reservoir to the sample cups are necessary to prevent sample trailing. A single long wash cycle is not as effective as the three wash cups between samples.

4. Room temperature must be <25 °C. At higher temperatures the ratios of solvent, diluent, sample, and air in the extraction coils are affected, causing volatilization and rapid depletion of the solvent reservoirs. Best results are obtained when the temperature of the solvent solution in the ice bath is kept near 0 °C (a Styrofoam shipping box works well), and the room temperature is kept at about 22 °C.

5. The three glass pulse-suppressors in series for each of the solvent manifold lines serve two purposes: as surge suppressors and as solvent reservoirs. At the start of operation, each series is inverted, the first two suppressors are allowed to fill completely and the third is partly filled (leaving about 1 cm air space). In the upright position, the suppressors trap any air bubbles formed by volatilization of the solvent as it is pumped.

6. The type C membrane (Technicon) is inert to the solvent system and can be used for four weeks without changing. When necessary, the membrane is replaced according to standard procedures—i.e., presoaking in water and stretching. The inlet and outlet tubes of the dialyzer must be of Acidflex tubing on both sides. The frame is used in an inverted position and the donor (solvent) stream should enter the bottom side of the dialyzer. This allows gravity to aid in separating the aqueous and solvent phases. Inlet and outlet lines should be at the same level as the extraction and hydrolysis coils (Figure 4).

7. It is very important to keep transmission lines in the system as short as possible, especially where aliquots are removed from separators.

8. The lead end of the waste lines from each separator must be of large bore Acidflex (0.110 inch i.d., black), and the rest of the line of Teflon (0.095 inch i.d.). There must be a free flow, with no back pressure or excess siphon action on the separators. A frame is made to hold the C4 separators in position (Figure 4). The relative position of these separators and waste lines is adjusted to produce a smooth, regular flow and separation of the phases. The waste line for the C3 separator can be taped to the pump to maintain it in a vertical position.

9. After the priming samples are started, if any emulsion forms and is trapped in the lower section of the C4 separator, it can be washed out by quickly removing and replacing one of the aliquot lines at the
N5 nipple, allowing a small surge of air to be aspirated through the separator. After this operation, time must be allowed for a steady baseline to be re-established. If an emulsion continues to form in the separator, it indicates a malfunction. Examine all reagents, manifold tubes, connections and bubble pattern in the extraction section, room temperature, and the solvent temperature. A single trapped air bubble in the separator can be removed by tilting the separator until the aliquot line draws out the bubble.

10. The temperature of the oil heating-bath for cholesterol color development is set at the maximum attainable without causing the fluid to boil and surge (about 80 °C). Maintain a smooth flow in and out of the bath.

11. An undulating or irregular baseline usually indicates erratic pumping through one of the manifold tubes or a partly blocked connector. Even a "new" line will not always function properly. Manifold lines for color reagents should be checked first. Rarely, a whole package of tubes may not deliver consistently.

12. An excellent solution for removing protein and cleaning the extraction section is the biuret solution commonly used in the laboratory for total protein determinations.

13. The dilute hydrochloric acid used for the constant volume and wash cups (5 ml/liter) is superior to water or saline in providing a stable system and washout between samples.

14. Tygon pulse suppressors are used wherever aqueous reagents or air enter the system.

15. If there appears to be a drift in peak heights of the triglycerides with no concurrent drift in the baseline and no drift in peak heights of cholesterol, first check room temperature, then change the manifold tubes for color reagents for triglycerides.

16. Monitor the quality of the red Acidflex lines. If the surface of the tube is rough to the touch, then the volume delivery rate must be determined. This is particularly important for the largest size (PUR/WHT), which must deliver at least 3.2 ml/min; less will result in an emulsion forming during phase separation.

17. The importance of timing in connecting the extract aliquot lines at the beginning of operation and disconnecting them at the end of operation is very important. A stable, two-phase stream must be flowing through the separators at all times while the aliquot lines are connected to the system. Pumping aqueous solutions quickly deteriorates the Acidflex tubing. Accidental pickup of the aqueous layer in the cholesterol aliquot line may result in salt formation in the cholesterol heating bath, blocking the coil.

18. Values for patients' sera with lipid concentrations exceeding the span of the recorder may be approximated by analyzing after diluting with saline, but the resulting decrease in protein concentration affects the extraction, so that the analyzer value may appear increased by as much as 0.2 g/liter. A more nearly accurate determination can be made by diluting the high unknown with serum of known low lipid concentration. We use sterile calf serum for this purpose. If many unknowns are expected to be high, the span of the recorder can be recalibrated for higher concentrations.

**Results**

Reproducibility studies over a four-month period show that the system has an overall SD for triglycerides of 37 mg/liter (CV, 3.4%) and an SD for cholesterol of 34 mg/liter (CV, 1.8%). Table 1 shows examples of within-run and day-to-day reproducibility. The accuracy of the system was evaluated by comparing values so obtained with those reported for commercial control sera and specimens that were analyzed by other methods. Samples (n = 105) were analyzed by the proposed system and by a manual extraction procedure performed in another laboratory (Technicon Method No. N-70P). The concentrations
Table 1. Reproducibility of Cholesterol and Triglyceride Analyses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Within runa</th>
<th>Day to dayb</th>
<th>Mean</th>
<th>CV</th>
<th>%</th>
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<tbody>
<tr>
<td>g/liter</td>
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<td>g/liter</td>
<td>g/liter</td>
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<tr>
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<td>3</td>
<td>2.85</td>
<td>2.51</td>
<td>0.026</td>
<td>0.028</td>
<td>0.91</td>
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a Data compiled from values obtained with 20 samples at each concentration, run in sequence and in no special order.
b Data compiled from control pools analyzed during four months.
c Data compiled from an undiluted pool analyzed during two weeks.

Table 2. Results for 15 Samples by Our Method Compared with Those Obtained with a Manual Extraction Methoda

<table>
<thead>
<tr>
<th>Our method</th>
<th>Manual method</th>
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<tr>
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<td>g/liter</td>
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<tr>
<td>3.50</td>
<td>1.30</td>
</tr>
<tr>
<td>4.72</td>
<td>32.80</td>
</tr>
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</table>


Table 3. Results for Eight Samples by Our Method Compared with Those of a National Surveya

<table>
<thead>
<tr>
<th>Our value</th>
<th>Survey value</th>
<th>SDIb</th>
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<tr>
<td>g/liter</td>
<td>g/liter</td>
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<tr>
<td>1.59</td>
<td>0.76</td>
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<td>0.72</td>
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<td>0.72</td>
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<td>0.81</td>
<td>1.48</td>
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<td>1.52</td>
<td>0.86</td>
<td>1.55</td>
</tr>
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<td>0.71</td>
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</tr>
<tr>
<td>1.58</td>
<td>0.75</td>
<td>1.54</td>
</tr>
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</table>

b Standard Deviation Interval (±2.0 SDI is acceptable).

SDI = (our value - mean of comparative method)/SD of comparative method

of the samples ranged from 1.30 to 4.70 g/liter for cholesterol and 0.45 to 37.0 g/liter for triglycerides. The correlation coefficient (r) for results by the two methods was 0.98 for cholesterol and 0.99 for triglycerides. Table 2 compares some results by our procedure with those obtained by the manual extraction method. Our laboratory participates in the national survey conducted by the College of American Pathologists. Table 3 compares our results with those obtained by the reference methods of the survey.

This system produces linear results over a wide range. Figure 5 shows representative curves. For our requirements, the full scale deflection of the recorder is set for about 3.0 g of cholesterol and of triglycerides per liter. The standard curve is drawn on an overlay and the unknowns are read from the curve. Although a steady-state setting of the calibration should not be attempted because the high protein load would coat the extraction system, a satisfactory calibration can be achieved by sampling a serum standard repeatedly. Approximate values may be read directly from the chart. We have used recorder spans of up to 10.0 g of cholesterol and 15.0 g of triglycerides per liter.

Because primary standards cannot be used in this system, it is imperative to design a quality-control program that quickly reflects any malfunction. We have designed a statistical program which includes analysis of variance. Wall charts are used to show the

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daily analyzed values of control sera in terms of (a) the mean and standard deviation, (b) the average of replicates within run, and (c) the range of replicates within run. Malfunctions caused by reagents, instruments, or technician error can be defined and corrected within-run. At least two commercial control sera from different companies are used in the program as well as samples of pooled sera prepared in the laboratory. Specimens exchanged with other laboratories and participation in national surveys provide continuing evaluation of the accuracy of the system.

Discussion

Many procedures are being proposed for determining cholesterol and triglycerides in serum. Although the newer enzymatic procedures offer some advantages, many laboratories will find our proposed automatic extraction system less expensive in that the basic equipment is common to all continuous-flow chemical analyses and the reagents are those in common use and readily available. In the early development of the system, we used a Model I Technicon system with a fluorometer for triglycerides. When the Model II system and the 50-mm flowcell became available, we could design more reliable colorimetric manifolds.

The published reports of other investigators have helped us improve our system. For the triglyceride determination, we use the more stable reagents described by Foster and Dunn (2), and we have incorporated rhamnose in the reaction as described by Vaskovsky and Isay (3) to remove excess periodate for improved baseline stability and reproducibility. The ferric chloride color reagent (4) proved to be more stable for our cholesterol determinations. The proportion of glacial acetic acid was increased to solubilize the chloroform extract.

The aqueous stream from the dialyzer was collected, concentrated, and analyzed for both inorganic and organic phosphorus. None was detected. From this evidence and that of comparable triglyceride values with other methods, we deduce that the dialyzer membrane effectively separates the glycerol from the phospholipids in the extract. Operation of the system without potassium hydroxide produced no evidence of free glycerol or nonspecific reactants in the extract. The effects of bilirubin, hemoglobin, and glucose were examined by addition of excess quantities to analyzed sera. We found no significant changes in analyzed values for either cholesterol or triglycerides. We did not examine the effects of therapeutic drugs on the system. Although the cholesterol analysis could be performed with the extract from the first separation, we found much better precision and accuracy in the measurement if we analyzed the hydrolyzed extract after it had received an additional wash to remove more of the methanol.

The rate of analysis described is designed to provide a very high degree of precision. If some precision must be sacrificed to speed, one or more of the wash cups between samples can be eliminated, or a more rapid sampler cam can be used.

Data on lipids in serum are essential in the diagnosis and treatment of hyperlipidemias and in the investigation of lipid metabolism. Thus a technique such as the one described here is needed—one that requires a small sample volume, offers rapid analysis and precision, and can be used for pediatric patients, multiphasic screening, and small-animal research.

References