measured previously. Such measurements may provide insight into the lack of symptomatic hypocalcemia in the presence of the very low concentrations of ionized calcium often observed during transfusion with blood containing citrate and phosphate (1–4). The low cost to initiate this procedure should also be attractive for laboratories that regard high initial cost and cost per test of instrumentation for ionized calcium as excessive.

References

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An Optically Clear Hypercholesterolemic Hypertriglyceridemic Quality-Control Material Prepared from Animal Lipid Sources

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A hyperlipidemic control serum can be simply prepared from animal lipid sources. Beta- and pre-beta-lipoproteins containing cholesterol and triglyceride are removed from porcine serum by treatment with dextran sulfate and calcium ions. A triglyceride-rich fraction containing only trace amounts of cholesterol is isolated from chicken egg-yolks. The two fractions are then combined in 40 mmol/L sodium bicarbonate to give the desired values for cholesterol and triglyceride. The preparation is stabilized against surface denaturation during long-term storage at 5 °C, perhaps for as long as two years, by adding 0.25 g of Triton X-100 surfactant per liter, and against an accidental exposure to short-term freezing by adding 10 g of sucrose per liter. We used this solution as a diluent to reconstitute lyophilized bovine serum. The resulting product, having been prepared from only animal sources, is free of hepatitis-associated constituents, and is remarkably clear, homogeneous, and stable. Results obtained with it are precise.

The association of increased concentrations of certain serum lipid constituents and increased risk of heart disease (1, 2) dictates that the precision and accuracy of lipid assays be carefully monitored. Pooled specimens of lyophilized human or animal serum are commonly used to evaluate and assure analytical performance. Hyperlipidemic human sera are expensive to prepare and sometimes difficult to obtain.

Recently we examined 22 lots of commercial human-serum-based quality-control materials for several hepatitis-associated materials: HBsAg, HBsAb, HBcAb, and HAVAb (3). All were positive for one or more of these constituents.

In view of the health hazard involved in handling quality-control materials prepared from pooled human sera, we show how to prepare hyperlipidemic serum from animal sources, such serum is naturally free of hepatitis-associated constituents.

The concentration of lipids in the serum of herbivores such as horses or cattle, from which large amounts of serum can be readily obtained, is too low to be useful in monitoring lipid assays effectively (4). Because of the denaturation and insolubilization of beta- and pre-beta-lipoproteins during lyophilization, serum products with a high lipid content generally reconstitute slowly, and the reconstituted fluid is often quite turbid (5). The lack of clarity and the inhomogeneity related to the uneven dispersion of insoluble material may decrease the usefulness of these products (6). A simple way is needed to increase the triglyceride and cholesterol concentrations of animal sera without adversely affecting the reconstitution rate or clarity of the product.

Recently, we described a stable human lipoprotein diluent for use in reconstituting lyophilized human serum for the preparation of clear, hyperlipidemic quality-control materials.

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Here, we have modified this process: we prepared a stabilized lipid diluent containing isolated porcine lipoprotein and a triglyceride-rich fraction from hen eggs, for use in reconstituting lyophilized bovine serum to produce clear, hyperlipidemic quality-control materials with precise concentrations of lipids within the range useful in physiological measurements.

Materials and Methods

Analytical Methods

We determined total lipids by the method of Frings et al. (8). Cholesterol and triglycerides were measured, unless otherwise noted, by enzymic techniques (9, 10). We measured absorbances of lyophilized and reconstituted sera with an Acta III spectrophotometer and measured pH with an Expandomatic pH meter (both from Beckman Instruments Inc., Fullerton, CA 92634).

Preparation of Lyophilized Bovine Serum

Bovine serum (0.5 L) was separated from freshly clotted blood collected at a local abattoir and dialyzed against a 15-fold excess of a 1.8 g/L solution of sodium chloride at 3-5 °C for 18 h. The preparation was centrifuged (4500 × g, 10 °C, 10 min). We decanted the clear supernatant liquid from the small amount of precipitate, which analysis indicated was mainly fibrin and fibrin split products, and added 2.9 g of sodium chloride per liter. The bovine serum had a cholesterol concentration of 1.08 g/L, a triglyceride concentration of 0.15 g/L, and a total lipid concentration of 2.15 g/L. The serum was filtered through a 0.45-μm (av. pore size) membrane (Millipore Corp., Bedford, MA 01730), apportioned into 3.0-mL volumes, frozen, and lyophilized. The vials were sealed under reduced pressure and stored at 5 °C.

Preparation of Lipid Diluent from Porcine Serum

We separated 2 L of serum from clotted porcine blood, freshly collected at a local abattoir, and added 50 mmol of calcium chloride per liter and 0.5 g of dextran sulfate (Type 500-S; Sigma Chemical Co., St. Louis, MO 63178) per liter, to precipitate the beta- and pre-beta-lipoproteins (11). After it had settled for 1 h, the preparation was centrifuged (4000 × g, 10 °C, 15 min) and the supernatant solution was decanted.

We resuspended the lipoprotein precipitate with a 20-fold volume excess of 25 mmol/L calcium chloride and centrifuged the mixture (4000 × g, 10 °C, 15 min). The supernate was decanted and discarded. This process was repeated until the supernatant solution was free of visible hemoglobin. The lipoprotein precipitate was suspended in 45 mL of water. We added 12 g of barium chloride (final concentration, 0.25 mol/L), 0.65 g of disodium ethylenediaminetetraacetate (EDTA) (25 mmol/L), and 0.75 g of tetratosodium EDTA (25 mmol/L), to redissolve the lipoproteins. The pH was maintained at 6.9 by adding a 4 mol/L sodium hydroxide solution. After it was stirred for 1 h, the mixture was centrifuged (30 000 × g, 10 °C, 15 min). We decanted the clear lipoprotein supernate from the barium dextran sulfate precipitate and added 7.4 g of sodium sulfate (0.26 mol/L) to the preparation, to remove the excess barium. The pH was maintained at 6.9 by adding the 4 mol/L sodium hydroxide solution as required. After stirring the mixture for 1 h, we centrifuged it as before, decanted the clear lipoprotein supernate from the barium–dextran sulfate precipitate, and added 7.4 g of sodium sulfate (0.26 mol/L) to the preparation, to remove excess barium. If necessary, the pH was adjusted to 6.9 with the 4 mol/L sodium hydroxide solution. After 1 h, the mixture was centrifuged (15 000 × g, 10 °C, 15 min). The clear liquid was decanted from the barium sulfate precipitate and dialyzed twice against a 20-fold volume excess of 30 mmol/L sodium bicarbonate for 10 h at 5 °C. Any additional precipitate that formed during dialysis was removed by centrifugation. We diluted the clear lipoprotein concentrate to 0.5 L with a 40 mmol/L sodium bicarbonate solution. The final solution had a total lipid concentration of 5.4 g/L, a cholesterol concentration of 1.76 g/L, and a triglyceride concentration of 1.6 g/L.

Preparation of the Triglyceride-Rich Fraction from Hen Eggs

The yolks from three hen eggs were separated from the egg white and washed with a 10-fold volume excess of isotonic sodium chloride to remove any adhering egg white. We punctured the yolk membrane with a scalpel and collected the yolk fluid. The membranes were discarded.

Twenty-five milliliters of yolk fluid was diluted threefold with sodium citrate, 30 mmol/L. The pH of the mixture was adjusted to 6.8 with 1.0 mol/L NaOH. After 30 min the preparation was centrifuged (30 000 × g, 10 °C, 15 min). We decanted the supernate from the insoluble cholesterol-rich fraction and diluted it twofold with 30 mmol/L sodium citrate. After the pH was adjusted to 5.8 with acetic acid/water (equivalent mixture) the preparation was centrifuged (30 000 × g, 10 °C, 10 min). The oily supernate was collected and heated in a sealed flask to 65 °C for 60 min, to inactivate any enzymes present. The fraction was then cooled to room temperature and diluted twofold with the sodium citrate solution. We adjusted the pH to 6.8 with a 1 mol/L solution of NaOH, and after 30 min centrifuged the solution (30 000 × g, 10 °C, 15 min).

We decanted the supernatant solution and diluted the liquid twofold with the sodium citrate solution. After the pH was adjusted to 5.8 with the diluted acetic acid, the mixture was centrifuged (30 000 × g, 10 °C, 10 min) and 13 mL of yellow, oily supernate was collected, which had a concentration of 314 mg of total lipid per gram, 230 mg of triglyceride per gram, and only 15 mg of cholesterol per gram. No free glycerol was observed (12). Thus nearly three-quarters of the lipid appeared to be triglyceride, with only a little cholesterol. Thin-layer chromatography (13) indicated that the cholesterol present was about 90% free and about 10% esterified. This fraction is still usable after storage for a month at 5 °C, but then should be discarded. The concentrate remained soluble in dilute buffer solutions as described below during this period.

The concentrated triglyceride-rich fraction is readily soluble in dilute salt solutions such as 40 mmol/L sodium bicarbonate or 40 mmol/L sodium chloride at an alkaline pH in the range of 7.5 to 10.0. The preparation was directly added to the previously described porcine lipoprotein diluent to give a triglyceride concentration of 2.9 g/L. Because this fraction contains so little cholesterol and the porcine lipoprotein diluent contains more cholesterol than triglyceride, mixtures of the two fractions to give various concentration ratios of cholesterol to triglyceride may be readily prepared.

Stabilization of the Lipid Diluent

We added a sufficient quantity of the triglyceride-rich fraction from egg-yolk to the porcine lipoprotein preparation to produce a solution of desired cholesterol and triglyceride concentration. The diluent was protected from microbial degradation to some extent by addition of thimerosal (0.2 g/L), cycloheximide (actidione) (0.2 g/L), disodium EDTA (0.1 g/L), and tetratosodium EDTA (0.1 g/L). We also added 10 g of sucrose per liter, to help protect the diluent from denaturation if the mixture should inadvertently be frozen (7). And 0.25 mL of Triton X-100 surfactant (Rohn and Haas Co., Philadelphia, PA 19105) per liter to protect the lipoprotein from surface denaturation on long-term storage or shaking. The so-
Table 1. Effect of Triton X-100 on the Surface Denaturation of the Diluent Lipoprotein by Shaking

<table>
<thead>
<tr>
<th>Triton X-100, mL/L</th>
<th>Optical clarity (A710 nm)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.560</td>
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<tr>
<td>0.062</td>
<td>0.120</td>
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<tr>
<td>0.125</td>
<td>0.050</td>
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<tr>
<td>0.25</td>
<td>0.030</td>
</tr>
<tr>
<td>0.50</td>
<td>0.025</td>
</tr>
<tr>
<td>2.0</td>
<td>0.030</td>
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Solution was then filtered through a 0.45-μm Millipore membrane and apportioned into non-sterile vials, which were stoppered, sealed, and pasteurized at 70–75 °C for 1 h on two successive days. We stored the vials at 5 °C.

Results and Discussion

Prevention of Microbial Growth

After two months of storage at 5 °C, 0.1-mL aliquots from five vials were tested for the presence of viable organisms by inoculation onto Petri dishes containing the following media: tryptic soy agar with 50 mL of sheep blood per liter; and chocolate agar with enrichment (cat. no. P2300 and P2306; Gibco Diagnostics, Madison, WI 53713); thioglycollate broth (cat. no. 135C; Carr Scarboroug Microbiologicals, Inc., Decatur, GA 30035); Sabouraud dextrose agar and Lowenstein Jensen medium (cat. no. 11584 and 20909; BBL, Cockeysville, MD 21030); and brain heart infusion agar with 50 mL of sheep blood per liter, and mycobacteria 7H11 agar (cat. no. 0037-01 and 0838-01; Difco Laboratories, Detroit, MI 48232). The plates were incubated at 35 °C in a carbon dioxide atmosphere (50 mL/L), except for the Sabouraud dextrose agar (25 °C and 35 °C without carbon dioxide) and the brain heart infusion agar with sheep blood (35 °C, but no carbon dioxide). The mycobacteria 7H11 agar, Lowenstein–Jensen medium, Sabouraud dextrose agar, and brain heart infusion agar were incubated for four weeks, other media for five days. No growth was detected in any of the media.

Microscopic smears were prepared from each of the five vials with Gram and auramine rhodamine fluorochrome stains and examined for either viable or nonviable organisms. No organisms were observed in any of the Gram-stained smears. In smears from one of the five vials examined by auramine rhodamine fluorochrome stain, two to three acid-fast organisms per smear were observed. These organisms appeared to be one of the saprophytic species of mycobacteria commonly found in water supplies. Although these organisms were present in the sample vials, they did not multiply in any of the culture media. Because the antimicrobial combination mentioned above, plus pasteurization, evidently suffices to prevent growth, the diluent vials need only be filled under “clean” rather than sterile conditions.

Stabilization of the Lipoprotein Diluent to Surface Denaturation

Beta- and pre-beta lipoproteins are sensitive to denaturation by surface tension during long-term storage or by shaking, such as might occur with transport. Various amounts of Triton X-100 were added to the lipoprotein diluent, to reduce the surface tension, and the solutions were vigorously agitated with a mechanical shaker for 20 min. The results (Table 1) indicate that the surfactant helps stabilize the lipoprotein to surface denaturation during shaking, as judged from the optical clarity at 710 nm. Addition of 0.25 g of Triton X-100 per liter also affords good protection to the lipoprotein in the diluent during long-term storage at 5 °C, and does not interfere with lipid assays.

Analysis of Lipoprotein Diluent

The lipoprotein diluent was analyzed for 18 commonly measured constituents with a Technicon SMA 18/60 continuous-flow analyzer (14). Within the limits of sensitivity of the methods used, the diluent contained no glucose, urea nitrogen, chloride, potassium, uric acid, calcium, phosphate, “direct” bilirubin, total bilirubin, creatinine, aspartate aminotransferase (EC 2.6.1.1), lactate dehydrogenase (EC 1.1.1.27), alkaline phosphatase (EC 3.1.3.1), or creatinine kinase (EC 2.7.3.2). Small amounts of total protein (2.0 g/L) and albumin-reacting protein (1.0 g/L) were measured. Sodium, 41 mmol/L, and bicarbonate, 37 mmol/L, from the sodium bicarbonate added to the diluent, were also observed. These analyses were performed to determine the approximate amount of the commonly measured clinically significant constituents in the diluent. The suitability of this approach as a comprehensive control must be determined from a more extensive analysis of diluent/serum-component interactions.

Storage Stability of Lipoprotein Diluent and Lyophilized Serum

To study the effects of accelerated aging on stability, we incubated samples of both the lipoprotein diluent and ly-
ophosphatase serum base for one and two weeks at 37 °C. The contents of the vials containing serum were then reconstituted with 3.0 mL of a similarly incubated vial of diluent and evaluated for optical clarity, total lipids, cholesterol, and triglycerides (by enzymic and nonenzymic methods); the results are shown in Table 2.

The reconstituted incubated samples retained their optical clarity, and their total-lipid, cholesterol, and triglyceride concentrations were identical with the original material, within the analytical error of the methods. We have been successful in storing samples at 5 °C for six months for use in total lipid, cholesterol, and triglyceride assays. Longer-term storage of the lipid control components at 5 °C has not been evaluated, but the results on accelerated aging suggest that both the lipoprotein diluent and lyophilized bovine serum base are stable for perhaps as long as two years at 5 °C.

Previously, we obtained standard deviations of 0.076 g/L for 6.54 g/L of total lipid, 0.045 g/L for 3.207 g/L of cholesterol, and 0.029 g/L for 2.019 g/L of triglyceride, on replicate analysis of the same vial of lipid control (7)—values only slightly lower than the overall inter-vial precision for total lipid, cholesterol, and triglyceride observed with the animal-source lipid control. This technique for preparing hyperlipidemic sera from animal sources produces a stable material, free of hepatitis-associated constituents, and with remarkable clarity and uniformity. The alternative use of such animal-based quality-control materials in instances where a human-serum-based product is not required may allow for the conservation of the limited resource of human blood components for more essential needs and reduce the exposure of laboratory workers to possible infectious material.

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References